RECEPTOR-MEDIATED ENDOCYTOSIS OF LOW DENSITY LIPOPROTEINS IN AORTIC ENDOTHELIAL CELLS

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David Austin Sanan

Institute for Electron Microscopy
South African Medical Research Council
Cape Town

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Certificate of Supervisor

In terms of paragraph G.P.8 of Rules for the Degree of Doctor of Philosophy, I, as supervisor of the candidate, **David Austin Sanan**, certify that I approve of the incorporation into this thesis of material that has already been published or submitted for publication.

Dr. G. A. Coetzee. 14th April, 1986.

Senior Lecturer in the Department of Medical Biochemistry.

ABSTRACT

Lipoprotein binding and metabolism in actively-dividing (subconfluent) and quiescent (postconfluent) bovine aortic endothelial cells (ECs) were qualitatively investigated by fluorescence microscopy using dioctadecylindocarbocyanine-labelled lipoproteins and by indirect immunofluorescence microscopy. LDL and acetylated-LDL (AcLDL) were seen bound to the surfaces of subconfluent ECs (at $4^{o}C$ or at $37^{o}C$), as a random distribution of punctate foci. ECs therefore closely resembled fibroblasts in the distribution of LDL receptors on their surfaces. No binding of LDL was seen on postconfluent EC surfaces by either direct or indirect fluorescence microscopy. The patterns of AcLDL binding on postconfluent ECs resembled those on subconfluent ECs. Intracellular LDL and AcLDL occurred as perinuclear accumulations of large fluorescent disc-shaped profiles in subconfluent ECs. These accumulations were shown to arise from surface-bound material by pulse-chase experiments. Intracellular LDL was absent in the majority of postconfluent ECs, while AcLDL accumulation was massive. "Wounding" of cultures allowed simultaneous assessment of lipoprotein metabolism in quiescent and actively-dividing areas of the same culture.

Quantitative assessments of the above-mentioned phenomena were made using ^{125}I-labelled lipoproteins. Receptor-mediated binding of LDL decreased five to ten-fold as the cultures modulated from subconfluent to postconfluent morphology. No receptor-bound LDL was detected in postconfluent ECs. Conversely, the amount of AcLDL bound increased at least five-fold during EC growth in parallel cultures. The amounts of lipoproteins endocytosed and metabolised were generally related proportionately to the amounts bound in each case.

The distribution of LDL receptors on cultured cells was also investigated at the ultrastructural level using colloidal gold-conjugated LDL as a probe, and similarly labelled antibodies as probes. Whole-mounted cells with receptor probes bound to them were examined directly in the transmission electron microscope. The topographical distribution of LDL receptors has not been investigated by these techniques before. A novel method of preparing cytochemically-labelled, whole-mounted cells from styrene culture dishes was developed and used in this study. LDL Receptors expressed on the surfaces of human skin fibroblasts served to standardise these colloidal gold techniques and fortuitously led to new information on receptor distribution. Normal (FGo) and LDL receptor-negative mutant fibroblasts (GM 2000) acted as positive and negative controls respectively. Normal fibroblast LDL receptors were grouped into clusters consistent in size with coated pits (200 - 500 nm in diameter). A novel finding was the presence of a diffuse population of receptors scattered randomly amongst the clustered receptors. Another mutant fibroblast, GM 2408A, known to have an aberrant LDL receptor distribution, was also examined. Its receptors were shown to be dispersed singly, and in occasional groups of two and three, at random over the cell surfaces. No clusters were detected. The receptor-negative GM 2000 bound virtually no probes. While not as sensitive as the colloidal gold-conjugated LDL probe, an antireceptor monoclonal antibody (IgG-C7), localised by indirect immunogold labelling, gave similar results when applied to the above cells. This was taken as strong corroborative evidence that the LDL receptor distributions as determined by colloidal gold-conjugated LDL were correct. It is suggested that the dispersed population of receptors on normal fibroblasts may represent newly-emerged recycling receptors which have yet to cluster in coated pits.

A further new finding reported here is the existence of the same two patterns of LDL receptors, dispersed and clustered, on the surface of subconfluent ECs. It was noted, from the study of whole-mounted and thin-sectioned cells, that the receptors were preferentially arranged in rings following the circumference of coated pit areas on the cell surface. Often these rings associated in groups or even coalesced into compound clusters. The significance of these groupings is not yet understood. In sharp contrast to the situation on subconfluent ECs, no LDL receptors (probed with the extremely sensitive colloidal-gold conjugated LDL) could be detected at the EM level on the surface of postconfluent ECs. Active cells in wounded postconfluent monolayers expressed abundant receptors detected at the EM level.

It is concluded that postconfluent quiescent bovine aortic ECs in vitro metabolise virtually no LDL via the LDL-receptor pathway due to a vanishingly low number of LDL receptors. This contrasts with the ability of postconfluent cells to metabolise relatively large amounts of AcLDL via a receptor-mediated mechanism. The significance of these conclusions is discussed with respect to the interaction of plasma lipoproteins with the endothelium in vivo.

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ABBREVIATIONS

AcLDL ..... Acetylated LDL
AcAcLDL ..... Acetoacetylated LDL
AHF ..... Antihaemophilia factor
AHG ..... Antihaemophilia globulin
Apo B ..... Apolipoprotein B
A.R. ..... Analytical reagent
AuLDL ..... Collodial gold-conjugated LDL
BSA ..... Bovine serum albumin
DFM ..... Direct fluorescence microscopy
DGDW ..... Double glass-distilled water
DiI ..... 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate
DiI-LDL ..... DiI-labelled LDL
DiI-AcLDL ..... DiI-labelled acLDL
EC ..... Endothelial cell (ECs, pl.)
ECDGF ..... Endothelial cell-derived growth factor
EC-LDL ..... LDL modified by endothelial cells
EDGF ..... Endothelial cell-derived growth factor
EDTA ..... ethylenediaminetetraacetic acid
EGF ..... Epidermal growth factor
EM ..... Electron microscope
EV ..... Endothelial vesicle
FCS ..... Foetal calf serum
FGF ..... Fibroblast growth factor
FITC ..... Fluorescein isothiocyanate.
GAG ..... Glycosaminoglycan
HEPES ..... N-2-hydroxyethylpiperazine - N'-2'-ethanesulphonic acid
HDL ..... High density lipoproteins
HIFCS ..... Heat-inactivated foetal bovine serum
HUVEC ..... Human umbilical vein endothelial cell
IDL ..... Intermediate density lipoproteins
IEL ..... Internal elastic lamina
IIFM ..... Indirect immunofluorescence microscopy
Kd ..... Equilibrium dissociation constant
LDL ..... Low density lipoproteins
LDL-R ..... Low density lipoprotein receptor
LM ..... Light microscope
LM-ARG ..... Light microscopic autoradiography
LPDS ..... Lipoprotein-deficient serum
MDA-LDL ..... Malondialdehyde-modified LDL
MDGF ..... Macrophage-derived growth factor
Me-LDL ..... Methylated LDL
MEM ..... Eagle's minimal essential medium buffered with bicarbonate
MEM-LPDS ..... MEM containing LPDS
MEM-HEPES ..... MEM containing LPDS and buffered with HEPES
MEM-FCS ..... MEM containing FCS
MgPBS ..... Phosphate buffered saline supplemented with 2mM Mg^{2+}
PBS ..... Phosphate buffered saline
PDGF ..... Platelet-derived growth factor
RAHFVIII ..... Rabbit antihuman factor VIII
RME ..... Receptor-mediated endocytosis
SEM ..... Scanning electron microscope
S.D. ..... Standard deviation
SMC ..... Smooth muscle cell
TCA ..... Trichloroacetic acid

TC-LDL ..... Tyramine cellobiose-labelled LDL
TC-Me-LDL ..... Tyramine cellobiose-labelled methylated LDL
TEM ..... Transmission electron microscope
Tris ..... Tris-hydroxymethyl amino methane
VLDL ..... Very low density lipoproteins
vWF ..... von Willebrand Factor
WPB ..... Weibel-Palade Body
β VLDL ..... beta-migrating VLDL

CONTENTS

CHAPTER ONE

General Introduction and Review

AIMS

The overall aim of this thesis was to examine the dynamics of low density lipoprotein (LDL) receptors in vascular endothelial cells (ECs) undergoing density-dependent morphological changes as they matured to form classic contact-inhibited monolayers in cell cultures. The monolayers closely resembled, both morphologically and functionally, the endothelium of an artery like the aorta. In the normal healthy artery, the endothelium forms the frontier between tissues and blood: it efficiently regulates the uptake of plasma LDL in accordance with metabolic needs. Because ECs present a barrier to circulating, possibly atherogenic, lipoproteins in vivo, it was important to investigate directly the activity and location of LDL receptors in convincing models of the normal and wounded endothelium. In this way, the behaviour of LDL receptors in normal and wounded endothelium in the presence of atherogenic lipoproteins might be better understood. Also, because of their modulation from an active, proliferative state to a quiescent state, ECs present an excellent system in which to follow LDL receptor dynamics at high resolution in the electron microscope, from the viewpoint of cell biology. Therefore an investigation was made of the receptor-mediated uptake of LDL by actively-dividing (subconfluent) and quiescent (postconfluent) foetal bovine EC in culture. In addition, postconfluent cultures were mechanically wounded: LDL interactions with regenerating cells at the wound edge were compared with undisturbed monolayered cells.

In the present investigation, the interactions between LDL and ECs have been monitored using biochemical and morphological methods. These included the use of ^{125}I-labelled LDL to quantitate binding, endocytosis and degradation of LDL; while light microscopical visualisation of fluorescent LDL and electron microscopic cytochemistry of gold-labelled LDL were used to monitor structural features of the interactions of LDL with ECs.

Normal and mutant human skin fibroblasts, the LDL receptors of which have been investigated intensively by others[reviewed 101, 95] for over ten years, were used as controls in the present investigation. Because of their ease of isolation and cultivation, fibroblasts were used for almost all the work which led to the elucidation by Goldstein and Brown in 1974[36] of the receptor-mediated pathway of low density lipoprotein endocytosis and degradation and its role in plasma cholesterol homeostasis. The implications of their discoveries have vastly increased the understanding of the role played by lipoproteins in health and disease, ranging from the grossest manifestations of lipoprotein disorders at the clinical level, to the subtlest level of the genetic control of the expression of lipoprotein receptors *. This knowledge has had enormous impact on the management and treatment of lipoprotein disorders, notably the genetic condition known as familial hypercholesterolaemia (FH). The availability of mutant fibroblasts with deficiencies in lipoprotein receptor expression was crucial to the analysis of binding, internalisation and recycling of LDL receptors and to the analysis of synthesis and expression of new receptors.

An appreciation of the structure and function of the aortic endothelium is essential in the study of atherogenesis, the process of development of atherosclerosis. Atherosclerosis is a serious vascular lesion leading to ischaemic heart disease, cerebrovascular accidents (strokes) and peripheral vascular disease. Current thinking about mechanisms of atherogenesis centres on the interactions amongst circulating lipoproteins, bearing triglycerides and cholesteryl esters, and vascular cells including EC, monocyte-derived macrophages, neutrophils and smooth muscle cells. Genetic and dietary causes of aberrant lipoprotein metabolism which directly affect plasma levels of atherogenic lipoproteins are of central importance. The sites of atherosclerotic lesions involve the endothelium directly. Endothelial integrity must be

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* The fertile mixture of medical, genetic, cell and molecular biological, biochemical and morphological knowledge in the creative minds of J.L. Goldstein and M.S. Brown led to their receiving the 1985 Nobel Prize in Medicine.

disturbed for an atheromatous lesion to exist, according to the most complete theory of atherogenesisis - "The Response to Injury Hypothesis".[224, 225] This hypothesis postulates the primacy of the endothelial lesion in the aetiology of atherosclerosis.

The study of atherogenesis in vitro offers clear advantages over in vivo models, the most important of which is greater control of experimental parameters. The critical evaluation of such model systems must be based upon knowledge of the normal and pathological structure and function of the endothelium. In the wide range of cells currently used as physiological models, cultured ECs are remarkable in retaining many of their in vivo biological characteristics, which are reviewed below. The review will include a discussion of the role of the endothelium in atherogenesis.

Endothelium

Definition

The endothelium is a monolayer of smooth, flattened, epithelioid cells of mesenchymal origin forming the inner lining of all blood vessels, the heart, lymphatics, the cornea and the meninges. Endothelia occur in all vertebrates and in other phyla such as annelids, molluscs, arthropods, echinoderms and hemichordates.[51]

Although the endothelium may at first appear to be a mere histological feature of other more complete organs, it should properly be considered an organ in its own right. It is a diffused rather than a discrete organ; "unique only because of the way it spreads throughout the body in the form of a ubiquitous lining of blood vessels".[78] The organ is composed of a single kind of cell, the EC, uniquely endowed with a panoply of differentiated functions. These cells form the interface between circulating blood and tissues where they have a clear boundary function. This unique "frontier" location demands that ECs be adapted to carry out all their biological functions, e.g. mitosis, whilst remaining firmly anchored with their luminal surfaces exposed to flowing blood under pulsatile pressure. Besides forming an antithrombotic, rheologically efficient boundary, they are actively involved in haemostasis, macromolecular transport and exchange, hormone release and signal transduction, the complement system, enzyme release, vasoactive substance release as well as many metabolic and secretory activities.[143] The growing list of newly-discovered EC functions defines an organ of vast structural and functional complexity necessary to cope with the transport logistics of the entire body.

Etymology

W. His formally used the term "endothelium" in 1865, but its practical use probably pre-dated this.[1, 273] The most plausible derivation of the term may be as a variation on the theme "epithelium" *. Much controversy, in histology circles, followed W. His' introduction of the term "endothelium" and its use was opposed until after 1930. The dispute centred on the necessity for another term when epithelium would have sufficed. In 1935 Kohn argued that since the endothelia were of different embryological origin to other types of epithelia, the term endothelium was justified and should be retained.[1]

Embryology and Angiogenesis[81, 289]

Endothelium originates from splanchnic mesoderm via mesenchymal aggregates laid down along the lines of future vessels. ECs are thus related to the connective tissues which are also of mesenchymal origin. Most other epithelia are derived from the ectodermal or endodermal germinal layers and are embryologically different from endothelia.

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* ETYMOLOGICAL FOOTNOTE.

The term epithelium is ascribed to Henle, who reputedly used it in 1830,[273] but another source[1] suggests that F. Ruysch may have coined this term even earlier, in 1716. Epithelium (from Greek: epi = upon, thele = nipple) means literally "upon the nipple"[56] and probably describes the cells covering the papillae (literally "little nipples") of the corium.[1] In deriving the term "endothelium", "endo" means "inner" and relates to the location of the endothelium on the luminal surface of all blood vessels. The "-thelium" part of the term, as suggested above, probably mimics the ending of "epithelium" , the general term encompassing cells specialized as surface layers. Fair comment on this hybrid term came from anatomist J. Hyrtl, who in 1878 wrote: "Whoever found such a contradicting name: he really does not know what epithelium vi nominis means. Endothelium is an etymological monstrosity. A literal translation would mean : in the wart (nipple)."[273] One noteworthy departure from this etymology was that of W. Hueck who suggested, alternatively, that "-thelium" could derive from the greek word "thelos" meaning a cover.[1] This neatly and consistently explains the compound term "endothelium" as "endo" (inner or inside) plus "thelos" (cover or lining) as meaning "inner lining".

The development of all blood vessels begins with capillary generation from ECs, a process known as angiogenesis.[81] The regeneration of tissues (healing) and tumour growth both involve angiogenesis and are therefore fertile fields for the study of EC differentiation. The ontogeny of ECs is a cardinal embryological event because most developing tissues must be vascularised for continued growth. In the vertebrate embryo, after gastrulation, mesodermal cells differentiate and migrate out from the primitive streak thus creating a third germinal layer between the endoderm and ectoderm. Splanchnic mesodermal cells migrate centrifugally along predetermined routes of the major vessels leaving islands of haemangioblastic tissue in various stages of differentiation distributed throughout the entire embryo. Those islands furthest from the primitive streak (which is on the midline) differentiate into vessels first, so the development of the vasculature appears to be centripetal. Haemangioblastic islands have EC primordia on the periphery and haematopoetic cells in the centre: these regions develop separately to produce ECs and blood cells respectively. The islands anastomose with one another along the routes of the major blood vessels establishing patent capillaries before blood flow starts.[81] A likely mechanism of lumen formation involves the alignment of ring-shaped cells to form seamless segments of a patent vessel. This is called intracellular canalisation because the lumen forms within the cell itself. Evidence supporting this mechanism includes the existence of seamless ECs in nascent capillaries and the formation of ring-shaped cells by capillary ECs in culture.[289]

Side branches form by outgrowth or "sprouting" of solid cords of EC from the new capillaries or small vessels. These sprouting cords become patent either by intracellular or intercellular canalization. Intercellular canalization may involve the generation of the new vessel lumen by the ECs, which join either with themselves or their neighbours thus enclosing a roughly cylindrical space. The precise steps in these mechanisms of lumen formation are still unknown.[81]

Initially, the distribution of haemangiogenic islands or masses delineates the adult vascular tree morphopoetically. Then capillaries form and link up to form the framework of the tree. From these capillaries the major vessels are elaborated by the addition of non-endothelial cells, which produce the medial and adventitial layers. Veins and arteries differentiate by some as yet unknown process which probably involves haemodynamic factors. The more peripheral plexuses and capillary beds develop in response to local angiogenic forces in the tissues and their ontogeny is independent of the major morphopoetic ground plan.[289]

Angiogenesis may depend on humoral factors released by cells other than ECs.[reviewed 81] Klagsbrun et al. have isolated and purified a mitogen which has the capacity to stimulate capillary but not aortic bovine ECs.[154] This is the first specific mitogen for capillary ECs and is therefore important to the understanding of angiogenesis.

Structure of the Endothelium[reviewed 149]

The circulatory system, both blood and lymphatic vasculature, is completely lined by ECs disposed to form a simple squamous epithelium called the endothelium. Endothelium is subsumed under the epithelial category because it is a sheet-like aggregation of closely-apposed polyhedral cells lining the surface of a body cavity.

The endothelium may form the entire vessel, as in the case of capillaries, or just the innermost (luminal) layer of the intima of larger vessels. ECs are differentiated into phenotypes which are structurally and functionally adapted to the physiological demands of the tissues served by different segments of the vascular tree. While it is clearly beyond the scope of this introduction to describe this great phenotypic variation in detail, salient features distinguishing arterial from capillary ECs will be discussed.

Capillary Endothelial Cells

In 1661 M. Malpighi discovered capillaries in the frog mesentery and lung. His observation provided the theoretical link between arteries and veins which completed William Harvey's theory of blood circulation.[273] Nowhere are ECs more active than in the capillaries, which they alone constitute. Here the ECs mediate the tissue exchanges vital for all cells in multicellular animals with circulatory systems. The capillaries can be classified into three types: continous, fenestrated and sinusoidal. The total surface area of the capillary network is about 6000 m^2 - a vast physiological exchange area resulting from arborization.[56]

Continous capillaries, like those of the myocardium, have 2-3 ECs in a typical cross-sectional profile. The cells are held together by tight junctions, have no perforations but do have a basal lamina (basement membrane) on their abluminal fronts.[149] **Fenestrated** capillaries have transcellular pores occluded by delicate semipermeable diaphragms. They are found in kidney, intestine and endocrine glands, all sites of extensive chemical exchange between blood and tissue.[149] **Sinusoidal** capillaries have greater diameters than either continuous or fenestrated capillaries (30-40μm as opposed to 7-9μm). There are large openings between the cells through which interstitial fluid has direct access to the blood. To aid this, EC basal laminae are discontinuous. Sinusoidal ECs occur in the liver, spleen and bone marrow where macromolecular and cellular traffic between blood and tissue is particularly heavy.[149]

Endothelium of Large Arteries

Muscular arteries comprise three major layers of which the innermost, or **intima,** is endothelial. The tunica intima is composed of a luminal monolayer of ECs lying atop the subendothelium, a basement membrane in which is embedded the internal elastic lamina. The monolayered ECs rest upon a basement membrane which they secrete on their abluminal front. The cells are closely-apposed, polygonal and elongated in the same direction as the blood flow. There are no fenestrations but occasional holes exposing the subendothelium do occur.[183, 156] The subendothelium contains intimal smooth muscle cells.[21] In atherosclerotic arteries, this region may be invaded by large numbers of SMCs from the underlying medial layer.[224, 225, 223] The tunica media or **media,** is composed of multilayered smooth muscle cells with their long axes arranged circumferentially, interspersed with the connective tissue proteins elastin, collagen (Types I and III) and proteoglycans. These proteins compose the **extra**cellular matrix which is secreted by the mesenchymal SMCs. In the larger arteries, **an** external lamina separates the media from the next adventitial layer. The tunica adventitia, or **adventitia** contains fibroblasts embedded in a tough matrix of elastic fibres and collagen Type I. Vasa vasorum supply blood to the media and adventitia of the larger vessels since metabolite exchange with the blood in their own lumina via the endothelium would be too limited.

Since cultured aortic ECs have been used in this study to model aspects of the intima in vitro, a consideration of aortic ECs as they occur in vivo (in the intima) will be be instructive. Large arteries are susceptible to atherosclerosis therefore the cultured ECs under discussion are necessarily those which originate from large vessels and which resemble their in vivo counterparts as closely as possible.

Topography

Amongst the techniques that have been used to describe the topography of the aortic endothelium are surface replication in conjunction with light microscopy;[1] conventional scanning electron microscopy of coated preparations[103, 66] and uncoated preparations;[211] and high resolution replication in conjunction with transmission electron microscopy.[53] The description of the surface of aortic endothelium which follows is largely based on these studies.

Early morphologists used the elegant "Hautchen" (German for little skin, pellicle or membrane) technique to strip selectively the endothelial monolayer from the exposed intima of large vessels such as the aorta. These ""Hautchen"" could be mounted on glass slides for

histological examination by light microscopy. Lord Florey[79] and others[208, 209] used a variety of histological stains, including silver, on these "Hautchen" preparations to enhance the intercellular boundaries of the ECs. They were able to see clearly the shape and arrangement of ECs under normal and pathological conditions.

Silver metal has been known to deposit out from silver nitrate solutions and to stain intercellular clefts at the margins of ECs in situ. The technique was used by Zahn as long ago as 1884.[208] Poole and co-workers[209, 208] used it to study long-term regeneration of mechanically damaged endothelium in rabbit aortae. The interpretation of the morphological changes accompanying the re-endothelialization of denuded areas of aorta depended on the cell margins being clearly delineated by silver stain *. That silver does outline cells by binding heavily in the marginal regions was unequivocally established by Buck[39] and Florey et al.[79] using transmission electron microscopy on sectioned material. Silver grains were found to coat all exposed parts of the EC membranes but superposition phenomena could explain why, in en face light microscopy, the cell boundaries seemed to be selectively stained. More recent studies using scanning electron microscopy have confirmed these transmission findings.[91, 211] Fryer et al.[87] showed that fibroblast cell margins also stained positively with silver salts so the reaction was not specific for ECs margins. Nevertheless, the stain could be used to delineate the characteristic cell boundaries of ECs.

The "Hautchen" technique may cause damage to the endothelium during the stripping procedure, which is a serious disadvantage particularly in pathological investigations. Scanning electron microscopy, in which the endothelium is viewed in situ and at higher resolution, solves this problem but creates some of its own. Studies have been made of the silver-stained normal rabbit aortic intima[103, 66] using scanning electron microscopy. Images showed confluent sheets of rhomboidal cells with nuclear bulges, orientated with their long axes in the direction of blood flow. Cell sheets were devoid of fissures or holes. In contrast silver-treated endothelium appeared abnormal when judged by transmission electron microscopy.[53] Another SEM study[211] compared silver-stained rat aortic intima with uncoated, unstained osmium thiocarbohydrazide osmium (OTO) treated preparations. Structures previously described as stomata £ were resolved by as extensive overlapping of the edges of adjacent ECs, as previously described by transmission electron microscopy of sectioned material.[246] Resolution of the surface of endothelium by conventional SEM preparative techniques is disappointing as the heavy metal coating can obscure fine details such as caveloae which are visible in TEM studies. Better results have been attained with uncoated endothelia (see OTO technique - above)[211] but the inherent resolution of the SEM is limiting when compared with that of the TEM. Excellent resolution of surface features can be obtained by viewing replicas of freeze-fractured[233] or critical point-dried ECs in the TEM.[249] Minute details like overlapping cell margins, microvilli and caveolae are easily resolved.[53, 249, 233, 234]

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* Sinapius[258] questioned the use of silver stain for this purpose claiming that silver lines unrelated to EC margins were seen in his preparations. Poole made a very strong case for the continued use of the technique, when, by careful standardization of the staining procedure, he was able to limit the silver reaction to the endothelial monolayer alone. Stain penetration to deeper layers of the intima produced artefacts which led critics to doubt whether the stain indeed revealed EC outlines.[208]

£ So-called stomata were seen in endothelial "Hautchen" preparations by Auerbach in 1865. These were islands of cellular material (in later studies outlined by silver stain[211]) much smaller than ECs. These rare structures (frequency: 2 per 1000 cells) were explained as gaps allowing transport of particles through the endothelium or channels guarded by flap-like cellular extensions.[211] In a recent paper, Majno et al. examined silver nitrate stained rat aortic endothelium by transmission electron microscopy of serial thin sections. Stomata were found to correspond to myoendothelial herniae.[183]

Glycocalyx and Electrochemical Microdomains

The luminal surface of the endothelium is the glycocalyx which largely consists of proteoglycans, with external glycosaminoglycan (GAG) side chains. There are other glycoproteins associated with the EC surface including sialoglycoproteins and receptors, e.g. for LDL and insulin. The major glycosaminoglycan (GAG) on EC surfaces is heparan sulphate, which extends negatively-charged groups externally. It may contribute to the antithrombotic properties of the EC surface, as may the binding of heparin. Lipoprotein lipase is thought to be bound to the endothelial cell surface via heparan sulphate "receptors". The detailed biochemistry of the glycocalyx of the EC is still unknown.[44] GAGs and other surface molecules are responsible for functional differentiation of domains on the surface of ECs and other cells. Surface molecules of varying charge, in different combinations, probably provide mechanisms for the selective binding, sorting and gating of ligands and other macromolecules selectively taken up by the EC.[256]

The Simionescus and co-workers have used electron-dense probes of differing size and charge to gather information on the electrochemical properties of the capillary endothelial surface at the ultrastructural level.[256] Anionic microdomains on pancreatic capillary ECs were found on the fenestrae and to a lesser extent over the coated pits while the plasmalemma was relatively free of them.[254] Liver sinusoidal capillary ECs, on the other hand, have cationic and anionic sites mixed in their coated pits.[92] The microdomains of aortic ECs have not been mapped.

Antithrombotic Surface

The surface of the endothelium is rendered antithrombotic by production of prostacyclin (PGI_2, a potent inhibitor of platelet aggregation),[293] plasminogen activators[163] and vasoactive amines.[36]

Anticoagulant properties are also conferred on ECs by the heparin-like surface GAGs to which antithrombin III can bind. The endothelium synthesizes thrombomodulin, which with protein C has anticoagulant properties; and it maintains a low level of Tissue Factor, a potent coagulation stimulant with Factor VII.[197]

Endothelial Vesicles

Endothelial vesicles (EVs), usually called plasmalemmal vesicles, are reliable ultrastructural markers for ECs of all kinds, and particularly capillary ECs. EVs from frog mesentery capillary ECs have a mean external diameter of 90 nm[84] as measured from 14 nm hyperthin sections. As seen in conventional ultrathin sections (60-90 nm thick), EVs appear as spheroidal membrane-bound vesicles (outer diameter 60-80 nm), free in the cytoplasm and also fused by necks with the plasmalemma forming flask-like invaginations.[256] The necks have diameters ranging from 30 to 70 nm as measured in hyperthin sections.[84] Palade, who first described EVs, named them plasmalemmal vesicles because their membranes were continuous with the plasmalemma.[256] But perhaps the term plasmalemmal vesicle is misleading in the light of newer knowledge. Experiments based on the labelling of plasmalemmal surfaces with markers indicated that so-called plasmalemmal vesicles may not form by pinching off from the plasmalemma, but rather exist inedependently as a separate system of shuttling vesicles which temporarily fuse with either cell front.[256] Apparently during these fusion events, no exchange of membrane components with the plasmalemma occurs so that the vesicles retain their identity. Decoration of the stomatal rims of caveolae (open EVs) with the polyene antibiotic filipin suggests a possible compositional difference between the plasmalemmal and vesicular membranes,[256] which may contain more cholesterol. Thus EVs may originate independently of the plasmalemma and may be a discrete population of specialised transport vesicles.

There are three structural models for the capillary endothelial vesicular system.

1. A discontinuous pathway between the luminal and abluminal fronts of the ECs composed of EVs free in the cytoplasm and in various stages of fusion with one another and with the the plasmalemmae on both fronts was proposed by Bruns and Palade.[37, 38] Transport experiments by Clough[57, 58] in frog capillaries using ferritin to mark the vesicle populations have supported this theory. The rate of transport by such a system would be a function of the concentration gradient across the barrier formed by ECs. This rate would be limited, and possibly regulated, by the rates of vesicular fission and fusion events amongst the population of EVs.

2. Patent transendothelial channels consisting of fused vesicles might connect the luminal and abluminal fronts of ECs thus creating hydraulically conductive pores. Transport through such a system would be passive and driven by concentration gradients.[256]

3. Bundgaard and Frokjaer-Jensen[43, 85] have suggested that complex luminal and abluminal invaginations of racemose, interconnected clusters of EVs might temporarily or permanently fuse, creating transient and some small number of permanent transendothelial channels. This model allows for temporary connections between invaginations open to the extracellular spaces but does not support the Palade model of an intermediate population of free, shuttling vesicles. While not discounting the EVs as the system of large pores predicted by physiologists for macromolecular transport across endothelia, Frokjaer-Jensen suggests that the sessile invaginations of clustered EVs may have other more important functions, e.g. membrane storage and secretion.[85] These ideas are vigorously opposed by the Simionescus who present evidence of the trancytosis of lipoproteins by EVs in rat systems (see Transcytosis, p 23).[280, 257] The protagonist of this theory, Frokjaer-Jensen, claims that when EV profiles are examined in hyperthin sections (14nm thick) they appear as complex clusters always open on either cell front.[85] Ultrathin sections analysed by conventional transmission electron microscopy are inadequate for the three-dimensional analysis of EV ultrastructure according to Frokjaer-Jensen. Vesicular necks and interconnections are often poorly resolved or even missed completely because the section thickness is too great. As a result a higher proportion of vesicles may appear free in the cytoplasm than is actually the case.

The use of tracers such as ruthenium red[54] or tannic acid mordant techniques[43, 290] has established that the majority of vesicles, even apparently free cytoplasmic ones, are continuous with either cell front in aldehyde-fixed material. A recent high voltage electron microscopical study of tannic acid mordanted vesicles in rat mesenteric muscle capillary ECs showed showed free vesicles and clusters of interconnected vesicles open to the surface.[290] Apart from the inconsistency of free vesicles, this supports Frokjaer-Jensens's structural model.

All these models are subject to the limitations of ultrastructural analysis, particularly the artefactual generation of caveolae in aldehyde-fixed material.[175] Fixation may not instantly immobilize membrane elements, which may explain why rapidly-frozen unfixed aortic ECs had 70% fewer caveolae than glutaraldehyde-fixed rapidly-frozen material.[175] The racemose clusters seen by Frokjaer-Jensen[85] and Wagner and Robinson[290] may be artefacts brought about by accumulated fusion events starting near the plasmalemma where the fixative concentration is highest and continuing as vesicles from relatively aldehyde-poor deeper regions approach the plasmalemma. The inevitable variations in fixation procedures may explain the sometimes contradictory results obtained for endothelial vesicle ultrastructure. Gross overestimation of open vesicles may explain why physiological models of transport are at odds with structural ones.[175]

Weibel-Palade Bodies

Weibel-Palade bodies (WPBs) were first described in 1964[292] as new cytoplasmic components of unknown function but characteristic of ECs. Now 20 years later, they are associated with the storage and processing of von Willebrand Factor (vWF).[288] WPBs are ultrastructural markers for the ECs of man, pig, mouse, and rat in vivo and in vitro.[125] The organelle is not found in rabbit or bovine endothelia although, controversially, its presence was noted in bovine aortic ECs in the first report of their routine culture.[26] WPBs are membrane-bound, elongated cytoplasmic vesicles (0.6 x 2-3µm) with truncate ends, composed of parallel 15 nm diameter tubules longitudinally arranged and closely-packed in a relatively dense matrix. The tubular structures appear to have a substructure with a central core.[292]

Cytoskeleton[reviewed 25]

Since the EC is uniquely positioned at the boundary between flowing blood under pulsatile pressure and immobile subendothelium, the maintenance of a flat, rheologically efficient shape and firm adhesion to its substratum , particularly during migration or mitosis, are essential. The control of cell shape, motility and attachment all involve elements of the cytoskeleton. EC have the three cytoskeletal filament systems common to all non-muscular cells: 6 nm microfilaments, 10 nm intermediate filaments and 24 nm microtubules. The three systems are integrated into a microtrabecular lattice forming the cytoskeleton and motor system which acts in a coordinated way during cell spreading in cultured bovine aortic ECs.[12]

In ECs in general, **microfilaments** occur close to the abluminal plasmalemma in long, thick bundles up to 50µm long which may be periodically cross-banded with electron dense material. F-actin is the major component of the microfilaments but myosin and tropomyosin are present in small quantities. The presence of myosin and actin strongly suggests a motor function for the microfilaments.

Intermediate filaments , mainly composed of vimentin, occur in the ECs of most vertebrates. They are arranged in very thick bundles of 500-1000 filaments. Bundles or single filaments may be arranged as perinuclear rings or may be distributed randomly throughout the cytoplasm; but they are not associated with the plasmalemma. Cultured bovine aortic ECs have perinuclear intermediate filaments. Confluent monolayers have increased vimentin

synthesis rates.[25] One of the functions of intermediate filaments is the positioning of organelles.[25]

Microtubules radiate throughout the cytoplasm of ECs. During mitosis in guinea pig ECs, the mitotic spindle inserts diametrically across an annular intermediate filamentous scaffold. This may help to keep the mitotic plane parallel to the subendothelium and the direction of haemodynamic shear forces. In this way, adhesion is minimally disturbed during cytokinesis. Other functions of microtubules include cell migration and vesicular and organellar transport. EC microtubules are disassembled as a result of treatment with colchicine or vinblastine. This causes the intermediate filaments to cap, but leaves the microfilaments undisturbed so cell shape is unaltered. Lysosomes and other organelles are redistributed, mitosis stops, migration and secretion cease.

Extracellular Matrix Proteins

The extracellular matrix of the endothelium is commonly called the basement membrane and it contains inter alia: laminin, fibronectin, collagen, proteoglycans, von Willebrand Factor, and probably thrombospondin.[192] Lung derived EC cultures have been shown to produce elastin.[45] Collagen Types I-V are found in EC basement membranes in different combinations and proportions depending on the type of EC which secreted them. The adhesion protein laminin interacts with collagen Type IV which is the predominant type in basement membranes. The other four collagens are present in lower proportions. Type III collagen is present as fine fibrils in relatively high amounts in thrombi and aortic subendothelium. The fibrillar form of Type III collagen is highly thrombogenic. Both fibronectin and von Willebrand Factor bind strongly to fibrillar collagen.[237]

Intercellular Junctions

According to a freeze-fracture study, rat aortic ECs in situ have composite intercellular junctions. Belt-like occluding or tight junctions surround communicating or macular gap junctions to give tightly interconnected and intercommunicating cells.[255] Zonulae adherentes and desmosomes are not found in the junctional complexes of the arterial endothelium.

There has been much controversy concerning the existence of an intercellular cement substance which is selectively stained by silver nitrate. But this has been resolved by electron microscopic studies in which no separate cement substance could be detected.[79, 39]

The low degree of permeability of the endothelium to macromolecules such as horseradish peroxidase[264] was explained on the basis of the high degree of continuity of the tight junctions.[255] The small pore system postulated by physiologists for the transport of water and small solutes across endothelia is thought to be constituted mainly by transendothelial channels, fenestrae and intercellular junctions[256] (see Transcytosis, p23).

The von Willebrand Factor and Endothelial Cells

The endothelium and subendothelium are essential components of the system controlling haemostasis or blood coagulation. Cultured ECs retain many properties in vitro which relate to haemostasis.

Haemostasis or blood coagulation[18] is a response by the organism to prevent blood loss. It has three phases: a vascular response (vasoconstriction), platelet plug formation and the formation

of a fibrin clot at the site of vessel trauma. Insoluble fibrin is formed in the clot from its precursor, fibrinogen, at a rate proportional to the concentration of the proteolytic enzyme thrombin. Thrombin also activates Factor XIII which stabilizes the fibrin clot by cross-linking the fibrin fibrils into a matrix. The formation of thrombin depends on the activation of Factor X by either the extrinsic or intrinsic coagulation pathways. The latter is activated by platelet contact with thrombogenic fibrillar collagen of the subendothelium.

Most details of the intrinsic coagulation pathway have been elucidated in vitro but it is unclear how these apply in vivo. Nevertheless, as a result of thrombogenic contact of platelets (with glass) in vitro, Hageman Factor (Factor XII) is activated and this activation is amplified by the kallikrein system. Active Factor XII in turn starts a cascade of activations, each of which amplifies, the procoagulant response. This cascade follows the sequence: IX (Christmas Factor), Factor VIII and Factor X. Activated Factor X, in the presence of platelet phospholipids, calcium cations and Factor V, converts prothrombin to thrombin. Thrombin then acts, as described above, to form a blood clot.

Procoagulant Factor VIII is also known as antihaemophilic globulin (AHG) or factor (AHF). It is reduced or absent in patients with classic haemophilia A. Factor VIII is also associated with an autosomal dominant condition which results in von Willebrand disease, a haematological disorder of the intrinsic coagulation pathway where platelets bind inadequately to the subendothelium at sites of vascular injury. The cause is thought to be the absence of, or defects in, a Factor VIII-associated protein variously known as von Willebrand Factor (vWF), Factor VIII-related antigen and Factor VIII-associated antigen. It appears that the procoagulant form of Factor VIII and vWF form moeities of a complex Factor VIII which has both procoagulant and platelet-aggregating functions.[281] The vWF, a glycoprotein containing about 13% carbohydrate, forms by far the major portion of the FactorVIII/vWF complex.[201] It exists as a multimeric protein of disuphide-linked subunits of molecular mass 220 000 daltons. The multimers are themselves composed of variable numbers of protomers which are most commonly dimers or tetramers.[230] The maximum molecular mass of a multimeric molecule is of the order of 20×10^6 daltons.[174]

The vWF is synthesized by most ECs[24, 140, 145, 194] and also by megakaryocytes, and hence platelets. The vWF binds to both platelets and subendothelium coating both components which adhere in the binding response. A possible mechanism for this binding reaction could be the affinity of the vWF primed surfaces for one another, given the propensity of vWF to form large multimeric complexes.

By indirect immunofluorescence and immunoelectron microscopy, vWF was found to be concentrated in Weibel-Palade Bodies*. It has been suggested that the function of these bodies might be the final processing and storage of vWF.[288] Mature vWF arises after processing from a profrom of 275 000 daltons via pre-Golgi compartments, where dimerization and initial glycosylation take place, and then via the Golgi compartment and Weibel-Palade Bodies, where further carbohydrate processing, multimerization and prosequence cleavage are carried out.[287]

In the face of all this detailed information concerning vWF synthesis, the site of procoagulant Factor VIII synthesis remains obscure. Preliminary studies strongly implicate hepatic sinusoidal ECs.[18, 201]

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* Recently a second protein, also deficient in patients suffering from von Willebrand disease and named von Willebrand Antigen II or vW AgII, has been isolated. This protein is distinct from vWF, but like it, is synthesized by ECs. vWF and vW AgII levels vary proportionately in von Willebrand patients and both proteins are clearly implicated in the platelet binding response.[174] The two proteins are co-localized in the Weibel-Palade bodies as shown by immunofluorescence microscopy.

Endothelial Cell Movement

ECs are motile both in vivo and in vitro. Repair of deliberate intimal wounds in vivo depends on migration during the inital phase of 24 hours. Only after this does mitosis occur[266] (see Regeneration, Chapter 3).

Cultured human umbilical vein endothelial cells (HUVECs) undergo energy-dependent contraction and shape-changes after site-specific thrombin stimulation. This is most likely due to the polymerization of G to F-actin and reorientation of the filaments. Thrombin may contract endothelium during haemostasis thereby increasing intimal permeability.[90] This may in some way initiate atherogenesis by allowing atherogenic lipoproteins to enter the intima.

The Role of the Endothelium in Atherosclerosis

Atherosclerosis

Arteriosclerosis is hardening of the arteries. One manifestation of this serious condition is atherosclerosis, the final stage in the progressive development of atheromatous plaque in the arterial intima. The atheromata or fatty lesions which compose plaque are initially largely composed of yellow cholesteryl ester-filled foam cells originating from monocyte-derived macrophages.[221, 95] The development of these early lesions, or fatty streaks, is thought to be reversible by the process of reverse cholesterol transport from the macrophage/foam cells by high density lipoproteins.[33] Atheromata develop into fibrocellular or fibrous plaque which consists of foam cells (mainly originating from smooth muscle cells but some from macrophages), free cholesterol and cholesterol ester in a soft gruel covered by a fibroelastic capping of extracellular matrix proteins, fibrin and smooth muscle cells. Fibrin appears relatively late in the development of the lesion and is not considered an initiating factor. In the final stages of atherosclerosis, the plaque becomes complicated and hardens by calcification.

Fibrocellular plaque narrows the lumina of arteries causing ischaemia.[221, 229] Certain parts of the arterial tree are more susceptible to plaque build-up; the ostia of side branches, bifurcations, and curves where turbulent blood flow is likely to occur, are preferential sites for eventual plaque build-up[156] which tends to have a focal rather than a uniform distribution. Susceptible areas readily take up the dye Evans Blue, indicating their increased permeability. These areas are termed "blue" areas as opposed to "white" areas which do not take up dye. The coronary arteries are particularly vulnerable to atherosclerotic stenosis; if they are narrowed by 80% or more, incapacitating or fatal myocardial infarcts are likely to occur.[229]

Atherosclerosis afflicts man, particularly in stressful, industrialised societies; it is the major contributing factor to coronary heart disease, strokes and peripheral vascular disease. Epidemiologists have identified a series of constitutive and environmental factors, both negative (risk) and positive (protective), which influence the onset, virulence and progression, and regression of the disease.[229] Accelerated atherosclerosis is found in individuals with genetic defects in the expression of lipoprotein receptors, e.g. familial hypercholesterolaemia (FH). Type III hyperlipoproteinaemia is also highly atherogenic and occurs in persons with abnormal Apolipoprotein E.

Theories of Atherosclerosis

The aetiology of atherosclerosis in middle-age involves cellular proliferation and not degenerative processes as was originally thought.[221] Theories explaining the mechanism of atherosclerosis on a cellular and molecular basis draw on the pathobiological interactions of SMCs, macrophages, ECs, platelets, lipoproteins, and extracellular matrices.[221] Most theories strongly implicate the excess cholesterol laid down in diseased arteries as being atherogenic. This cholesterol originates mainly from the low density lipoproteins circulating in the plasma in different concentrations depending upon the genetic and dietary status of the individual.[95] The means by which cholesterol breaches the endothelial barrier and enters the underlying intimal layers may be the key event in atherogenesis.

The **Lipid Insudation or Infiltration Theory** of atherosclerosis postulates that increased endothelial permeability coupled with elevated plasma lipid levels forces lipid into the intima with resultant atherogenesis. Smith and Ashall[261] found little difference between LDL concentrations in atherosclerotic and normal intima and concluded that infiltration of LDL is unlikely to be a primary atherogenic event. The theory also cannot explain the focal nature of the disease.[229]

The **Incrustation Theory** postulates thrombus formation as the atherogenic initiating event but evidence for increased fibrin levels in early lesions is lacking.[263, 229]

The **Monoclonal Theory** proposes neoplastic smooth muscle cell proliferation from clones of cells identifiable by their expression of specific isozymes of glucose-6-phosphate dehydrogenase. This theory fails to explain how lesions arise with higher frequency in specific regions of the arterial system.[229]

The **Response to Injury Hypothesis** enunciated by Ross and Glomset[224, 225, 221, 222] postulates that endothelial injury initiates atherogenesis under predisposing conditions, e.g. the presence in the plasma of high concentrations of atherogenic lipoproteins. The antithrombotic endothelial surface is remarkably resistant to the adhesion of cells or platelets. But, in the case of injury to the endothelium, the exposed subendothelium readily binds platelets which probably release a chemotactic agent for medial SMCs which migrate into the intima. Here the SMC modulate from a contractile to a proliferative and synthetic state in which they are capable of secreting an ECM containing inter alia, fibrillar collagen. The SMCs may also ingest atherogenic lipoproteins thus becoming foam cells within the ECM. Re-endothelialisation of the lesion and regression of the simple plaque will take place if intimal injury ceases. But in the case of chronic, repeated insults to the intima, the atherogenic cycle continues and exceeds the capacity of the endothelium to regenerate itself. It is also likely that premature senescence of constantly regenerating endothelium in atherosclerotic foci may result in impaired efficiency of healing.

Nature of the Injury

Endothelial injuries capable of initiating atherogenesis are diverse: deliberate mechanical denudation , high shear forces, and hypertension are all physical means of causing injury. Deleterious chemical agents include atherogenic lipoproteins, toxins, hormones, carbon monoxide, homocysteine and perhaps thrombin (see EC Movement, p 12). Immunological insults (such as autoimmunity) and viral attack may also injure endothelial integrity.[212] The damage may be visible (desquamation, separation of cell junctions) or subtle so that it can only be detected physiologically by altered endothelial permeability.

Results of Injury

Smooth Muscle Cell Responses.
Platelets adhere to exposed subendothelium (which contains fibrillar collagen) where they flatten out. Platelet adhesion to ECM is all that is necessary for release reaction for serotonin;[71] although it has not been established, platelet-derived growth factor (PDGF) release from the αgranules probably also takes place. Platelet adhesion in vivo is a necessary precondition for the migration of SMCs through the fenestrae of the internal elastic lamina into the intima, where they later modulate and proliferate. Although PDGF has not been shown in vivo in the plasma or at the level of the atheromatous lesion, it is released in vitro by platelet aggregation. PDGF is chemotactic for unmodulated contractile SMCs, and mitogenic for modulated, synthetic SMCs in vitro. A strong case can be made for its role in stimulating the progression of the SMC phase of the atherosclerotic lesion.[221]

Explants of SMCs from rat aortas 4-7 days after de-endothelialization grew faster than explants from control animals. These SMCs from injured aortas were also serum-independent for some weeks, seeming not to require PDGF.[118] It was suggested that the PDGF probably released in vivo had a persistent effect on the SMCs.

Other growth factors produced by cells involved in the atheromatous lesion are endothelial-cell derived growth factor (ECDGF) and macrophage-derived growth factor (MDGF), which are both mitogenic for SMCs. But neither factor has been assayed in vivo. PDGF, ECDGF and

MDGF may all play a role in the proliferative phases of fibrocellular plaque, but this awaits proof.

Macrophage Responses.

Macrophages (derived from monocytes) are involved as foam cells with atheromatous lesions in the earliest stages, the fatty streaks. Monocytes have been observed entering the intima at sites of increased permeability in hypercholesterolaemic animals. Here they may release MDGF to stimulate SMC proliferation. How macrophages become foam cells in vivo was difficult to explain as they do not take up excess LDL owing to the regulation of their LDL receptors by feedback inhibition. However the discovery that they possess so-called scavenger receptors for chemically altered LDL provided a possible explanation. Scavenger receptors recognise most forms of LDL which have been made more electronegative by chemical modifications which selectively remove positively charged groups from lysine and arginine residues, which are likely to constitute most of the ligand-site for the LDL receptor (see below and Chapter 3, Discussion). Uptake of modified-LDL via scavenger receptors is unregulated and leads to gross overloading of macrophages with cholesterol esters, so that they resemble foam cells in vitro.[33, 180, 181] Data indicating the existence of modified-LDL in vivo are still lacking, so the role of modified-LDL in atherosclerosis remains speculative, yet attractive. Candidates for in vivo modified-LDL are discussed in Chapter 3.

Macrophages also have receptors for atherogenic lipoproteins called βVLDL, the uptake of which results in foam cell formation in vitro[180, 181] (see below). Foam cell explants from atherosclerotic rabbits express both βVLDL and modified-LDL receptors.[205] The macrophage origin of foam cells in early atheromatous lesions is very likely in the light of their special capability for sequestering cholesterol esters in the second of a two-compartment system. The regression of macrophage/foam cell fatty streaks might also be explained by the resecretion by foam cells of cholesterol and Apo E which HDL might accept and carry away to the liver (see HDLc below).

Plasma Lipoproteins

Cholesterol Homeostasis

Atherosclerosis can be conceived of as an imbalance in plasma cholesterol homeostasis. This is most clearly illustrated by the accelerated atherosclerosis which occurs in persons suffering from familial hypercholesterolaemia who lack functional LDL receptors.[95] Hepatocytes, extrahepatic cells, intestine and the family of lipoproteins act in concert to regulate cholesterol supply and demand.

LDL is a plasma lipoprotein with a core of neutral lipids (cholesteryl esters and triglycerides) enclosed within a shell of apolipoprotein , phospholipids and cholesterol. Approximately 70% of plasma cholesterol is carried in LDL particles which have 80% of their cholesterol as esters, and the rest as free cholesterol. LDL have a density range of 1.019-1.063 g/ml; diameter range 20-25 nm and molecular masses ranging from 2 to 3.5 x 10^6 daltons, depending upon the variable lipid load. Apolipoprotein B (Apo B) exists in two forms Apo B-48 and Apo B-100. Apo B-48 has about half the molecular mass of Apo B-100. A fixed mass of Apo B-100 is found in LDL where it contributes approximately 500 000 daltons to the total particle. Apo B-100 is synthesized in the liver from which it is exported as part of VLDL.

Other classes of lipoproteins include very low density lipoproteins (VLDL), chylomicrons, and high density lipoproteins (HDL), which are similarly structured to LDL, but differ in size, density, and in the kinds and proportions of their chemical components.

VLDL are rich in endogenously synthesized triglycerides, and contain cholesterol, and Apolipoproteins B, C and E. The VLDL triglyceride moieties are hydrolyzed by lipoprotein lipase at the capillary endothelial surfaces in adipose tissue. The fatty acids released are either stored by adipocytes or used as substrate by muscle. The depleted VLDL become intermediate density lipoproteins (IDL) or VLDL remnants (containing Apo B and Apo E) which are cleared by the liver LDL receptors (for Apo B and E containing ligands).

Chylomicrons, rich in triglycerides containing dietary fatty acids and containing Apo B-48 and Apo E are produced in the intestine whence they find their way into the portal vein via the thoracic duct. Like VLDL, chylomicrons lose their triglycerides by the action of lipase in the capillaries. Chylomicron remnants are cleared by the the Apo E receptors of the liver.

Cholesterol originates from the diet or by de novo synthesis in virtually all cells. Dietary cholesterol is transported from the gut via chylomicrons which transmute into remnants and carry the cholesterol to the liver. Here the cholesterol is used for lipoprotein and membrane synthesis, and bile acid production. In the last case, the liver excretes excess cholesterol via the bile duct into the gut from which it is voided with the faeces.

The liver also exports, into the plasma, cholesterol incorporated into VLDL which transmutes into LDL, the main source of cholesterol in the plasma. The cholesterol content of LDL may be modified by contact with high density lipoprotein (HDL) where transfer proteins mediate cholesterol exchange according to mass action. LDL is taken up by receptor-mediated endocytosis and degraded in cells expressing the LDL receptor (LDL-R). Free cholesterol is converted to cholesteryl esters in a reaction catalyzed by acyl-coenzyme A : cholesterol acyltransferase (ACAT). The cholesteryl esters are stored in cytoplasmic droplets until needed.

Endogenous cholesterol synthesis proceeds in cells from mevalonic acid precursors via a rate-limiting step controlled by the enzyme 3-hydroxy-3-methylglutaryl CoA reductase (HMG-CoA reductase).

Cholesterol homeostasis on a cellular level has been elegantly explained by regulation of the LDL pathway in human fibroblasts.[95] The fibroblast responds to changing internal levels of cholesterol by regulating the number of surface LDL-Rs by synthesis or degradation; by regulating the synthesis of endogenous cholesterol and by esterifying and sequestering excess intracellular free cholesterol. If there is a demand for cholesterol, more receptors are synthesized and inserted into the plasma membrane; de novo synthesis is accelerated and cholesteryl esters are mobilised from storage. If there is an oversupply, these processes reverse by end-product feedback inhibition. These regulation processes have been studied in minute detail by Goldstein and Brown[96] who gave special attention to the structures of the LDL-R and HMG-CoA reductase, and to the coordinate control of these two crucial elements.

HDL have been implicated in a putative reverse cholesterol transport system which is theoretically required to explain how excess cholesterol is carried back to the liver from remote sites for excretion. HDL are capable of collecting cholesterol from cells in culture and precedent for this behaviour in vivo exists.[187] Nascent HDL particles arise from the liver as discoid protein-rich particles. They associate with an enzyme called lecithin cholesterol acyl transferase (LCAT) which catalyzes the conversion of cholesterol to cholesteryl esters which are sequestered in the core of the particle. HDL progressively loads with cholesterol esters becoming lighter and larger through the intermediate forms, HDL3, HDL2, and HDL1. Cholesterol exchanges can occur between HDL2 and other lipoproteins. HDL1 is also known as HDLc because it arises in cholesterol fed animals. HDLc is loaded with cholesterol and has also acquired Apo E which targets it either to the Apo E receptors (liver) or B,E(LDL) receptors (general), allowing rapid clearance. When the peripheral tissues of rabbits were cholesterol-loaded, HDL enrichment with cholesterol esters and Apo E was observed in vivo.[187] Thus a role for reverse cholesterol transport by HDL seems likely, qualifying these liproproteins as antiatherogenic since they lead to the clearance of possible cholesterol overload.

Lipoprotein interactions with the tissues depend upon EC mediation including lipoprotein lipase activity, lipoprotein transport, metabolism and possibly chemical modification. EC modified-LDL (EC-LDL) (see Chapter 3, Discussion) has been extensively studied in vitro and is the result of metal catalyzed peroxidation of LDL. Whether such a process could take place in vivo is a point for speculation. Certainly macrophages can produce free radical peroxides which can modify proteins for uptake via scavenger receptors, possibly ECs can do the same. ECs may be injured by their uptake and degradation of modified LDL, which is potentially atherogenic.

Elevated plasma levels of LDL are associated with accelerated atherosclerosis.[95] The cholesteryl esters in atherosclerotic plaques are derived mainly from LDL as evidenced by the high levels of linoleate esters which are characteristic of LDL.[31] High dietary intake of cholesterols leads, not directly, but via VLDL secretion to high plasma levels of cholesterol in the form of IDL. These high levels are capable of down-regulating the B,E (LDL) receptors which effectively retards the clearance of IDL. The resulting persistent IDL populations tend to convert to LDL (losing Apo E in the process) which then builds up in the plasma to produce a potentially atherogenic situation. It is not known why there is such a wide range of plasma LDL cholesterol levels in response to high dietary intake by individuals with normally functioning LDL-Rs. The liver may have genetically mutable mechanisms whereby a dietary cholesterol overload, having been taken up as chylomicron remnants, results in varying degrees of resecretion as VLDL.

If chylomicron remnants accumulate in the plasma they can also become atherogenic particles called βVLDL, along with VLDL remnants or IDL.[180] Cholesterol-rich βVLDL contain Apo E and are normally cleared by the Apo E receptor in the liver. Under circumstances in which the Apo E ligand is dysfunctional, βVLDL build up and are probably removed by macrophages which have a specific βVLDL receptor.

Apolipoprotein B

Apo B-100 is the ligand responsible for targetting the LDL to its ubiquitous receptors, termed B,E (LDL) receptors (LDL-Rs). The liver expresses the most LDL-Rs and is responsible for the catabolism of most plasma LDL, but the number of extrahepatic LDL-Rs is also considerable. Perhaps the highest density of LDL-Rs is expressed by adrenal glands, which with the ovaries, utilize exogenous (LDL-derived) cholesterol for steroidogensis. Apo B has proved difficult to characterise biochemically. The delipidated protein is extremely insoluble, vulnerable to protease attack and to cation-catalyzed oxidation. Attempts to define its subunit structure and composition, and to sequence fragements by conventional protein chemistry have yielded varied, sometimes conflicting, reports.[157] A molecular biological approach involving recombinant DNA methodology has led to the deduction, by a team led by W. Mahley, of the sequence of 1455 amino acids in the carboxyl terminus of the protein.[157] The thrombin fragment T2 from the carboxyl-terminal of Apo B represents 30% of the whole molecule. Following the sequencing of 29 amino acid residues from the amino-terminus of the T2 fragment, an oligonucleotide probe corresponding to the first 14 amino acids was synthesized and used to screen a library of cDNA clones from human liver. A clone designated p61D7 was used to probe for mRNA in human liver and HepG2 cells.

A 19 kilobase mRNA was found which coded for Apo B-100. This was sequenced for about 5 kilobases using overlapping cDNA probes. The 5 kilobase length coded for 1455 amino acids in the carboxyl terminus of Apo B. The amino-acid sequence was deduced and was free of internal repeats and homologies to other apolipoprotein sequences. The gene for Apo B was localised to the p24 region on chromosome 2.

The carboxyl-terminal region of Apo B had alternating hydrophilic and hydrophobic sequences consistent with a protein which interacts at regular intervals and alternately with lipid and aqueous phases. Potential glycosylation sites occurred at the transitions between hydrophobic and hydrophilic regions, where the carbohydrates would be favourably placed at a lipid/water interface. This fits well with the putative position of Apo B in the surface shell of the LDL particle.

A region rich in basic residues (lysines and arginines) was found; a strong candidate for the ligand region for the LDL-R which has an equal number of acidic residues. Apo E, which binds strongly to the LDL-R, has an homologous putative ligand site also rich in arginine and lysine residues. Evidence that these are indeed the ligand sites comes from the fact that chemical modifications of these residues abolishes the binding of Apo B and Apo E to the LDL-R. Heparin, which interferes with the binding of these apolipoproteins to their receptors, binds to the same region of Apo B.[157]

The LDL Receptor

The regulation of LDL-R activity in coordination with de novo synthesis of cholesterol, and mobilization or storage of excess cholesterol is crucial in plasma cholesterol homeostasis.[96] LDL are removed from the circulation mainly by the liver. In animal studies, it was established that about two-thirds of the plasma LDL was removed daily by hepatic receptors.[96] In addition to the liver cells, virtually all non-hepatic cells express LDL-R by means of which they obtain cholesterol for biosynthetic purposes.

Recently, a wealth of structural information relating to the LDL-R and to HMG-CoA reductase, the coordinate regulator of cellular cholesterol levels, have been published by Goldstein, Brown and co-workers.[reviewed 101, 96] These data are discussed below,with particular reference to LDL-R because they give valuable insights into the the regulation of plasma cholesterol levels. A fraction of mRNA, enriched for LDL-R-specifying mRNA, was isolated from polysomes of adrenal cortical cells using a polyclonal antibody against LDL-R. These mRNAs were used to generate a cDNA library which was screened with oligonucleotide probes coding for the amino acid sequence of a fragment of the LDL-R. Partial cDNA for the LDL-R gene was cloned and used to isolate an exon probe of the human LDL-R gene *. Pure human LDL-R mRNA was isolated with this exon probe and a cDNA for the entire 5.3 kilobase RNA sequence prepared. From the sequence of this cDNA, the amino acid sequence of the protein was deduced. A complex molecule with 860 amino acid residues arranged in five major domains was described.

Domain 1 (amino terminal) began with a 21 residue hydrophobic signal sequence which did not appear in the mature receptor which had 839 residues. This was followed by a sequence of 292 residues rich in cysteines arranged into seven or eight repeating microdomains. These 40 residue repeats had a series of acidic amino acids at their carboxyl terminals, which were complementary to the basic sequences, rich in lysine and arginine, thought to be the liganding sites of Apo E and Apo B. Each repeat had 6 cysteine residues which were all involved in disulphide bridges. Thus these repeats were probably highly contoured, rigid structures with multiple loops. Goldstein and Brown[101] speculated that these repeats could constitute multiple binding sites for ligands Apo E and Apo B. The stoichiometry of binding of Apo E with LDL-R is in accord with the above model of the multiple binding site, namely 4-8 Apo E molecules per receptor. It is interesting to note that only one Apo E-containing HDLc molecule binds to LDL-R for every four LDL molecules.[3] Since there is probably only a single Apo B molecule per LDL and multiple Apo E molecules on HDLc, this is also consistent with the receptor model. **Domain 2** contained 350 residues highly homologous with a sequence in the epidermal growth factor (EGF) precursor molecule, but not EGF itself. It was suggested[101] that LDL-R and EGF could have had a common ancestral gene for growth and nutrient supply coding for a membrane protein. Gene duplication and divergent evolution could explain their present independent existence. **Domain 3** contained 48 residues of which 18 were serines and threonines O-linked to carbohydrate chains. This domain could be situated immediately external to the plasma membrane where carbohydrate chains would help create a stalk, elevating the binding domains above the surface for easy ligand access. **Domain 4** was a hydrophobic membrane-spanning region containing 22 residues. Domain 5 comprised 50 residues of the cytoplasmic tail which was probably involved in directing the movements of receptors into coated pit regions of the plasma membrane.

Goldstein and Brown[101] have also categorized several naturally occurring mutations of the LDL-R into four classes. These are mutants

1) with no detectable LDL-R precursor molecule;
2) with defective LDL-R precursor processing, which do not appear on the cell surface;
3) with normally processed and externalized LDL-Rs but which have ligand-binding dysfunction;

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* The genes for LDL-R and Apo E reside on chromosome 19.

4) with LDL-Rs which fail to cluster into coated pits, but are otherwise normal.

One of the fibroblast control cell types used in this study (GM 2408A) falls into Class 4 (see Chapter 2).

Endocytosis

Endocytosis is any process by which cells take up extracellular particles, molecules which cannot cross the plasmalemma, and liquids in vesicles invaginating from the plasmalemma. Membrane recycling and cell motility are processes intimately connected with endocytosis.[31] Phagocytic cells, like macrophages, can ingest very large particles for degradation or transport and disposal. Phagocytosis (literally: "eating by cells") is a special case of endocytosis. Most cells, including ECs and human skin fibroblasts in culture, exhibit several kinds of endocytotic behaviour which involve structural and functional specializations suited to the uptake of lipoproteins.

Endocytosis may be adsorptive: molecules or macromolecules bind to the plasma membrane in some fashion, and by virtue of this binding are endocytosed. Adsorptive endocytosis may rely on specific, selective and concentrative binding of ligands to saturable high-affinity receptors, which are specialised membrane proteins, e.g. LDL-R, endocytosed via specialised regions of the plasma membrane. This is termed receptor-mediated endocytosis. Or it may rely on low-affinity adsorption to the plasma membrane which is taken up by pinosomes pari passu with liquids in a relatively non-specific manner.

Endocytosis may rely on pinocytosis (literally: "drinking by cells") or fluid endocytosis of liquids in pinocytotic vesicles or pinosomes. Davies[64] has measured rates of fluid endocytosis in cultured EC and related these rates to the different stages of growth. Contact-inhibited ECs had minimal rates of fluid endocytosis. In ECs pinocytosis is of special importance owing to the transendothelial transport (trancytosis) of material (e.g. LDL) via pinosomes or endothelial vesicles (EV).

Van Hinsbergh et al.[279] have estimated the relative contributions of the three types of endocytosis to the uptake of physiological concentrations of LDL by confluent human ECs in culture. Receptor-mediated routes contributed minimally. This revealed the predominant role of receptor-independent uptake and transport of LDL at physiological concentrations by ECs. These processes have a major impact on the delivery of lipoproteins to cells capable of degrading them (e.g. hepatocytes) and on the overall transport of lipoproteins in general. The normal function of trancytosis by ECs may be crucial to regulating the plasma levels of potentially atherogenic lipoproteins by mediating their transport to degradative sites.

Receptor-mediated Endocytosis

Apart from its primary importance in the regulation of plasma and cellular LDL levels, receptor-mediated endocytosis (RME) of LDL has provided a convenient system in which to analyse, from a cell biological viewpoint, ligand-receptor dynamics. Such an analysis has been underway for 12 years by Goldstein, Brown and co-workers.[reviewed 4, 101] The concept of RME grew from the realisation by these workers that aspects of the regulation of cholesterol in fibroblasts depended on three sequential events: binding, internalisation and degradation of LDL.[98] These stages were characterized biochemically and morphologically and have come to represent the paradigm for RME. Typical ligands that are taken up by RME are transport macromolecules (transferrin), nutrient macromolecules (LDL), hormones (insulin, EGF), immunoglobulins (IgG, IgA), viruses and macromolecules flagged for removal by scavenger systems, e.g. asialoglycoproteins and perhaps modified-LDL.

The Apo B-100 ligand of LDL, binds to LDL-Rs (see above) most of which are clustered above coated regions of the plasma membrane before ligand binding.[6] Receptors are not always preclustered, as in the case of the receptor for EGF. Unoccupied EGF receptors exist in a dispersed population, which clusters in response to ligand binding (ligand-induced clustering).[297] Different kinds of receptors may co-exist within the same coated pit which

appears to be indiscrimate: e.g. EGF, insulin, LDL and α2-macroglobulin receptors may co-localise within the same coated pits on fibroblasts.[185, 50, 282] Clustering of receptors could be a passive process driven by lateral diffusion in the membrane[15] or alternatively membrane flow from the leading edges of spreading cells (which usually have high rates of endocytosis) might aid the process.[31] There is evidence that a dispersed population of LDL-Rs co-exists with the preclustered receptors on the surface of cultured fibroblasts[7] (see Chapter 4). Robenek[215] however, believes that recycled receptors insert into the fibroblast plasmalemma as preclustered "plaques". Receptors bound to coated regions of the plasmalemma gradually draw together from an initial area of approximate diameter 500 nm to a tightly-packed aggregation approximately 200 nm in diameter. While this is happening, the coated region changes from a shallow depression to a basin-shaped pit and then to a flask-shaped invagination. This progressive invagination is an energy-dependent process thought to involve changes in the clathrin subunit (triskelion) arrays beneath the membranes.[4, 134, 259] These arrays form a basketwork lining of the pits on the cytoplasmic side of the plasmalemma.

The next phase is controversial: although the vesicle resulting from endocytosis of the coated pit is generally believed to be a coated vesicle, M. Willingham and I. Pastan[297, 299] dispute this in favour of their uncoated receptosome concept. Evidence favouring the first option centres on the appearance in the cytoplasm of profiles resembling coated vesicles with no apparent connections with the plasma membrane. The endocytosed ligand can often be clearly resolved within these coated vesicles. Willingham and Pastan argue that any coated structure resulting from endocytosis remains attached by a neck, often exceedingly thin and only visible in specially-stained serial sections.[298] Recent comment on this controversy came from Kolb-Bachofen[158] who claims the separate existence of coated vesicles in Kuppfer cells and receptosomes in liver sinusoidal EC.

The actual process of internalisation of the coated pit is extremely rapid: a "transfer" of the contents of an invaginated coated pit to a receptosome is said to take about 20 seconds. This may not be so much a transfer as a transition during which the membrane of the coated pit "balloons" out, leaving the clathrin coat behind. The uncoated vesicle then buds off into the cytoplasm and becomes a receptosome.[299]

A receptosome is an intermediate endocytic vesicle 200-300 nm in diameter; it has a clear lumen and usually a straightened region, with a somewhat fuzzy cytoplasmic coating, in its profile.[297] but there is virtually no clathrin present. The internal pH of approximately 5 is consistent with the ideas of Maxfield[63] who suggested that the mildly acidic interior of endocytic vesicles (receptosomes, endosomes) might cause the dissociation of ligand and receptor. The receptosomes can move, in saltatory fashion, from the peripheral cytoplasm to the juxtanuclear regions where prelysosomal and lysosomal compartments are found.[297] The receptosomes move along microtubular tracks in this rapid translocation to the vicinity of the prelysosomal apparatus variously described as GERL (Golgi-endoplasmic reticular lysosomal complex), CURL (compartment for uncoupling of receptor and ligand) or trans-reticular Golgi elements. Receptosomes then fuse with these vesicles. The trans Golgi vesicles also contain a population of small coated vesicles, which are distinct from those which appear near the plasmalemma. The former coated vesicles may have to do with receptor-ligand sorting. At the level of the CURL, a number of different fates may overtake the receptor-ligand complex, and these are classified into four different intracellular routes of receptor-mediated endocytosis by Goldstein and Brown.[101]

The "classic" route involves uncoupling of receptor from ligand in the endosome; sorting in the CURL; recycling of receptors to the plasmalemma; and degradation of the ligand. LDL, insulin, luteinizing hormone, asialoglycoproteins and α2-macroglobulin all follow this route.

Under certain conditions in vitro, this pathway may be short-circuited, and LDL and its receptor returned to the surface without involvement of the lysosomal compartment *.

The second route involves recycling of the ligand and receptor as typified by the transferrin system. The receptor-ligand complex does not dissociate but releases its iron in the acidic endosome. The complex avoids the lysosomal compartment and returns to the surface where less acidic conditions permit more iron loading.[101]

The third route, typified by the EGF system, involves degradation of both receptor and ligand after they have dissociated in the endosome.[63] There is no recycling of receptor. And finally, the fourth route is receptor-mediated transcytosis. There is no dissociation in the endosome and no lysosomal fusion. This route is followed by maternal IgG, secretory IgA, and recently insulin receptor-mediated transport has been described in endothelial cells.[152]

Goldstein and Brown[101] speculate that sorting and targetting of the different classes of receptors to the correct endocytic compartments probably depends on information carried by the receptors themselves. Specific "sorting" sites, distinct from ligand binding sites may exist. They speculate further that such sites might be multiple, carrying sequential information to direct the whole receptor itinerary. In this way, many different receptors, all taken up by the same coated pit endocytic shuttle, could each find their way along "predestined" endocytic routes.

Transcytosis

The transendothelial transport, or transcytosis of macromolecules which do not pass the endothelial intercellular junctions, has long been postulated.[38] Mouse aortic endothelium permeability was probed in vivo by the Steins[264] using peroxidases of two different sizes in an attempt to determine the exclusion limits of transendothelial routes. The larger probe, lactoperoxidase, was detected in the EVs but not the intercellular clefts. This work provided the first clear indication that macromolecules like high and low density proteins (HDL, LDL) were probably transported via the endothelial vesicular route.

Subsequently autoradiographic mapping of radio-iodinated VLDL, LDL, and HDL in rat aortic endothelium located these lipoproteins with high probability, in EVs.[266] Later work by Vasile et al.[280] established the transcytosis of exogenous LDL by directly visualising it (mordanting, immunoperoxidase) in rat aortic endothelium perfused in situ. The frequency of LDL particles in EV and subendothelial space provided quantitative evidence for transcytosis of most circulating LDL for use by other cells. The transcytotic route was responsible for low-affinity, non-saturable uptake and transport of LDL. Comparatively less LDL was processed via the endocytic pathway (coated pits, endosomes, multivesicular bodies (MVBs) and lysosomes). An interesting question regarding the above report concerns the amount of endogenous (rat) LDL which was bound to the aortic endothelium prior to experimentation. A low temperature washout followed by fixation, mordanting and electron microscopy would have established whether or not rat LDL was bound to coated pits. LDL metabolism in the rat is specialised and atherosclerosis is virtually unknown. The presence of high serum levels of HDL1 may be responsible for excellent plasma cholesterol control by reverse cholesterol transport in the rat.

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* RETRO-ENDOCYTOSIS

Aulinskas et al.[10, 9] have observed in human skin fibroblasts and smooth muscle cells a phenomenon which they call retro-endocytosis. This was a process whereby LDL was taken up and regurgitated, largely unchanged, after a short-circuit of the normal endocytic pathway. The LDL was released free and bound to receptor. The process only involved prelysosomal compartments of the endocytic cycle. The significance of this process is unknown and its existence in vivo has not been investigated. Speculating, it is not impossible that in macrophagoid cells, such a process may bring about chemical modification of proteins to "flag" them for later clearance via scavenger receptors.

In a further study[257] transcytosis and endocytosis of endogenous lipoproteins (probably LDL and VLDL) in vivo by capillary ECs in rats was directly visualised by transmission electron microscopy and quantitated by morphometric analysis. As in the previous study on exogenous lipoproteins, the transcytosis of LDL in these spontaneously hyperlipoproteinaemic, aged rats was greater than endocytosis and was selective for LDL-sized particles. Unfortunately, these findings in the capillary EC cannot answer the question of receptor-mediated endocytosis in the normal aortic EC in vivo.

There seems little doubt that, should there be a humoral overload of LDL, increased transcytosis of these particles will occur. Since the subendothelial LDL concentration in normal human aorta is twice that of the plasma,[261] transcytosis via EV may be an active transport process against this gradient. On the other hand subendothelial LDL may "partition" into the matrix with higher affinity than into plasma, thus providing the driving gradient for a passive process.

King and Johnson,[152] using a monolayer of bovine aortic ECs separating the two chambers of a transport vessel, have demonstrated receptor-mediated transcytosis of insulin. This intracellular route would correspond to the type 4 route of Goldstein and Brown[101] by which maternal IgG and secretory IgA are transported across epithelia. Receptor-mediated transcytosis of LDL across endothelia in vivo would be of doubtful relevance since receptor-independent transport already accounts for it.[280, 294]

Conclusion

After reviewing many of the properties and activities of the EC in vivo, the role of the endothelium in atherogenesis has been discussed in this introductory chapter. To place this properly in perspective, brief reviews of the plasma lipoproteins, receptor-mediated endocytosis of LDL, macrophage interactions with lipoproteins and finally plasma cholesterol homeostasis were also given.

Since the integral endothelium takes up and degrades normal and perhaps atherogenic LDL from the plasma, it was of interest to investigate the contribution of receptor-mediated endocytosis to the process. The Steinberg[267, 294, 47] and Simionescu[280, 257] groups have evidence for a small but significant receptor-mediated uptake and degradation of LDL into the aortic intima of rats and rabbits. But the mechanism whereby the endothelium controls the uptake of LDL via receptors has not been investigated in vivo. This mechanism forms the central question addressed in this thesis. What is the controlling factor in the inhibition of receptor-mediated endocytosis of LDL in a convincing model of the arterial endothelium? Is it the absence of receptors on the surface? Or is it the failure of the ECs to internalize receptor-bound LDL?

Receptor-mediated endocytosis of LDL occurs in cultured bovine aortic ECs. These cells modulate in culture from an active, dividing, motile state to a quiescent, contact-inhibited monolayer which mimics many properties of the endothelium. While active, ECs endocytose and degrade LDL via high-affinity receptors but while they are quiescent these activities are drastically reduced.

A longstanding model[284, 286] seeks to explain this behaviour by the inhibition of endocytosis of bound LDL. This inhibition was alleged to involve restricted movement of the LDL-R in the plasma membrane of contact-inhibited monolayers. However, in the present analysis of the dynamics of LDL-Rs by radiochemical, microstructural and ultrastructural methods, it appeared that LDL-Rs were expressed in vanishingly low numbers on quiescent monolayers. This observed reduction of receptor numbers may be explained by the classic Goldstein and Brown[32] model for LDL-R regulation which adequately accounts for the low uptake of LDL by high-affinity receptor-mediated uptake in quiescent EC monolayers.

Experimental Model

To answer the question of the control of LDL-R expression on the surface of the endothelium, a suitable experimental model was sought. Bovine aortic ECs in culture were chosen. The validation of this choice was based upon a thorough comparative review of the structure and function of ECs as they occur in vivo and in vitro. The culture model was then experimentally characterized before use in experiments.

Research Outline

ECs, in active and quiescent states, were used for the analysis of receptor-mediated endocytosis of 125I-LDL, fluorescent LDL (DiI-LDL) and colloidal gold-labelled LDL (auLDL). These methods permitted quantitative and qualitative (both microstructural and ultrastructural) statements to be made concerning LDL-R dynamics in an experimental model of the arterial endothelium. In addition, the ultrastructural probing of LDL-R distribution on fibroblasts and ECs has yielded some novel insights into receptor recycling.

CHAPTER TWO

Cell Culture

Endothelial Cell Cultures

The broader field into which this study fits concerns lipoprotein endocytosis and transcytosis in endothelia. These processes bear directly upon cholesterol homeostasis and atherogenesis which were discussed in the previous chapter. In the present investigation, the scope of the field was limited to receptor-mediated endocytosis of LDL and modified-LDL in active and quiescent endothelial cells (ECs).

Endothelial cells may be investigated in vivo,[294] in situ,[280] in organ[202] or in cell culture.[26, 148] This investigation demanded an EC culture model in which experimental manipulations could be quickly and reproducibly carried out without the varied, complex and even unknown factors obtaining in vivo or in situ. In a cell culture system, experimental control over growth medium composition, cell density, cell morphology, cell identity and lipoproteins could be rigorous. Experimental findings from cell culture systems would be subject to later confirmation in animal models in the final analysis.

The following properties were considered mandatory in the choice of cell culture system:

1) Cells should be of aortic origin.

2) Cells should retain, under culture conditions, as many in vivo characteristics of the endothelium as possible.

3) Cultures should be reproducible.

4) The system of culture should be simple and easy-to-handle during biochemical and morphological investigations.

In this chapter a review of the in vitro cultivation of ECs is presented. Upon this is based the choice of bovine aortic ECs as the cell culture system to be used in the experiments reported here. Arguments are presented to validate this choice of cultured bovine aortic ECs for the study of aspects of lipoprotein endocytosis.

Detailed methods for the isolation, cultivation and characterization of EC cultures used here are also presented. And finally, the characteristics of the cells used are presented and discussed.

The Development of Routine Endothelial Cell Culture Methods

Basic Culture Parameters

Simple, reproducible culture of ECs only became possible from the early 1970's. The exponential growth of biochemical and cell biological knowledge about ECs[142] has been the direct result of this development of routine culture technology. Increasing interest in the role of the endothelium in atherogenesis, haemostasis and thrombogenesis has also provided a spur to progress. A brief historical account of the development of cell culture methods for ECs is interwoven with a review of their in vitro characteristics.

In 1921 W.H.Lewis[165] reported the culture of ECs from chick embryo liver (in which ECs and parenchymal cells predominate). He was able to identify ECs in his cultures on the basis of their contrasting morphology compared to hepatocytes (neither cell type proliferated). He also produced EC explants from other embryonic tissues such as skin and skeletal muscle. On the basis of morphology, ECs could be identified as having migrated out from the capillaries of these tissues.[166, 167]

Simple explants, like those of Lewis, were used for the next three decades. The cells hardly proliferated at all and could not merit the name cell cultures. In vitro experiments up until the early 1970s were therefore based on tissue culture in its original sense:[124] small but whole pieces of vascular tissue were used to yield out-migrations of non-proliferative cells. Attempts to grow endothelium from cell sheets mechanically stripped from the vascular intima usually failed because of poor attachment to the substratum.[184] This stimulated the search for methods of dissociating dissociated the ECs before seeding into culture, in the hope that the single cells would attach more easily. In 1963, Maruyama[184] produced true EC cultures when he used trypsin to detach and dissociate intimal cells from human umbilical veins. These human umbilical vein ECs (HUVECs) did not proliferate but could be maintained in culture for approximately two weeks.

At this time, characteristics which allowed positive identification of ECs in culture had not yet been defined: so there was no way of detecting contaminants with a similar cultural morphology, e.g. other epithelioid cells. In addition, the purity of cultures could only be controlled by monitoring the source of the cells very carefully. After dissociating the endothelium by enzyme treatment, the vessels used were thoroughly checked for damage to the subendothelium, and cultures arising from suspect veins were discarded. Maruyama[184] was the first to culture well-attached, confluent monolayers of ECs from normal human umbilical veins. In the same year (1963) Pomerat and Slick,[207] using techniques similar to those of Maruyama,[184] isolated and maintained rabbit aortic ECs in culture. The cells supposedly proliferated, but the older cultures were mainly composed of spindle-shaped cells, as opposed to the flat epithelioid types seen in young cultures. Probably, the so-called growth was merely an overgrowth of contaminating fibroblasts or smooth muscle cells (SMCs) which are common in primary EC cultures.[245, 142]

In 1966, Fryer[87] used Maruyama's technique to grow ECs from human umbilical arteries. Arterial cells had to be collected from umbilical cords kept at 37°C because low temperatures contracted the arteries rendering them impossible to cannulate. He characterised his cultures by their ability to take up silver stain. Silver staining of the intercellular margins or "cement lines" was claimed to be an unique endothelial characteristic[1] but it occurred in other cell types as well[79] (see Chapter 1, p 6). Nevertheless, the deposition of AgCl and its development as silver metal does delineate cell margins extremely well, showing the shape of the cells and their arrangement in endothelial monolayers produced both in vivo and in vitro. The use of this stain persists amongst modern workers.[116, 91]

In order to reduce contamination by extraneous cells, Fryer like Maruyama histologically checked the vessels from which ECs originated in order to ensure that the underlying elastic

lamina (IEL) was undamaged. If damaged, cultures originating from that vessel were discarded. Large numbers of freshly isolated cells were needed to produce confluent cultures because no proliferation occurred rendering subculture impossible. Nevertheless, the quiescent arterial ECs were viable as evidenced by their phagocytic capacity. Fryer et al.[87] described a population of atypical cells which proliferated amongst the typical ECs in venous cell cultures. These cells might have been what later became known as "sprouters"[61, 77] (discussed below), but were more likely to have been contaminating SMCs or fibroblasts, or both.

Up to the end of the 1960s, the isolation of large numbers of relatively pure ECs and the maintenance of these in culture was possible. But the proliferation of ECs in vitro and their unequivocal identification were still goals to be achieved. From 1972-3 Jaffe,[148, 2, 146] considerably helped by the findings of Maruyama[184] and Fryer,[87] successfully isolated and propagated HUVECs. One of his major contributions was the use, instead of trypsin, of collagenase which selectively digested the subendothelial basement membrane leaving the internal elastic lamina (IEL) intact.[184] This treatment released intimal ECs but not cells beyond the IEL. In this way contamination of cell cultures by SMC was minimized.

Collagenase is a calcium-dependent proteolytic enzyme. It has been reported that very pure forms of the enzyme are relatively inefficient at releasing ECs from blood vessels compared with less pure forms.[142] After collagenase treatment, Jaffe collected cells in serum-containing fluids which appeared to protect the cells from further "collagenase" action. The numbers of viable cells were also increased by this method. Since serum contains inhibitors of serine proteases, to which class collagenase does not belong, it was suggested[142] that contaminating tryptic proteases might be responsible for the action of crude collagenase on ECs. With experience, Jaffe reduced the time of exposure of the tissue to collagenase from 45 to 15 minutes because prolonged enzyme treatment damaged the subendothelium allowing influx of fibroblasts and SMCs from the media. He also used carefully selected segments of umbilical cord undamaged by clamps, so that contamination by mechanically extruded SMC and fibroblasts was minimized.[147] But his crucial contribution was the use of foetal, not adult, bovine serum as a growth supplement to the EC media. For the first time, in vitro proliferation and therefore serial subculture (or passaging) became possible.

Gimbrone[93] analysed the contact-inhibition phenomenon in confluent HUVECs using tritiated thymidine to label nuclei active in DNA synthesis. He found that the rate of DNA synthesis was inversely related to cell density. Very few nuclei in contact-inhibited cell monolayers were labelled by tritiated thymidine detected by light microscopical autoradiography. He also found that, when quiescent cells were released from contact-inhibition by mechanical wounding, they became active and took up thymidine. Thus cultured HUVECs could be used as a model for the regeneration of damaged intima in experimental pathology.

In 1976, Haudenschild[126] reported the unresponsiveness of confluent HUVECs to serum growth factors. Most other cultured cells, even when confluent, responded positively to fresh serum by a spurt of growth characterized by migration and mitosis. In contrast, postconfluent contact-inhibited HUVECs did not respond to additions of fresh serum by migration, or mitosis which was monitored autoradiographically. Human skin fibroblasts and BALB/C 3T3 mouse fibroblasts responded in a dose-dependent way to fresh serum additions. Because ECs in vivo are continually bathed in serum and have a low mitotic index, it is not surprising that postconfluent HUVEC monolayers are resistant to the mitogenic factors present in serum.[126]

In 1975 a definitive paper on the culture of bovine aortic
ECs appeared.[26] They were isolated by Jaffe's collagenase procedure.[26] This was probably the first report of identifiable arterial ECs proliferating and being serially passaged in culture. The ease of culture and excellent retention of in vivo characteristics of bovine aortic ECs accounts for their continued use in many and varied experimental situations.[176, 245, 88]

Contaminants

Media commonly used for growing ECs were supplemented with serum, which contained platelet-derived growth factor (PDGF).[226] PDGF was mitogenic for the common contaminants of primary EC cultures, e.g. medial SMCs, but not for ECs themselves: thus contaminant growth was favoured.[88] To overcome the serious problem of contamination of bovine aortic ECs by SMCs, the selective uptake of radioactive (tritiated) thymidine by proliferating cells during DNA replication was exploited. Postconfluent primary cultures of ECs were incubated in media containing concentrations of triated thymidine lethal to dividing cells: only dividing SMC contaminants were killed by radioactivity ("thymidine suicide") while quiescent bovine aortic ECs survived.[77] But "thymidine suicide" was only partially effective, needing repeated applications to cultures. Atypical cells, termed "sprouters" (see below), which turned out not to be contaminants, tended to reappear with repeated passaging. Cloning proved a more effective means of obtaining pure cultures of ECs, although it did not necessarily rid cultures of "sprouters". This was achieved by seeding cells at limiting dilutions so that well-separated colonies of cells arose which could be assumed to have arisen from single cells.[198]

Sprouting

EC monolayers in vitro sometimes give rise to cells of altered morphology called "sprouters".[186, 244, 61] Sprouters occur as a secondary, netlike undergrowth in postconfluent bovine aortic ECs.[61] The shapes that sprouters assume are very varied: fibroblastoid rather than epithelioid morphology is the general form. Sprouters are so called because they superficially resemble capillary sprouts formed during angiogenesis.[11, 251] They are unlikely to be contaminants since they arise in cloned ECs. Sprouters are not contact-inhibited;[186] retain Factor VIII antigenicity as demonstrated by immunofluorescence ; and are ultrastructurally similar to ECs. They appear underneath the monolayer[61] and are separated from it by the basement membrane.[245] Cotta-Pereira et al.[61] never saw sprouter overgrowth of monolayers. They detected collagen type I and fibronectin production by sprouters. They considered sprouters to be phenotypically variant ECs which might be stimulated by growth factors in serum or by extracellular matrix (ECM). McAuslan et al.[172] reported variant bovine aortic ECs which produced sprouters growing both above and below the monolayer. The sprouting culture secreted three times as much fibronectin as monolayered cells, distributed over the upper cell surfaces and not only beneath the cells as in monolayers. Migrating variants left a trail of fibronectin, an activity which could cast these variants in the role of in vitro equivalents of angiogenic sprouts.

McAuslan et al.[171] identified growth factor signals (produced by ECs or present in fresh serum) capable of modulating the phenotype of a cloned bovine aortic EC variant (Sp.) into either fibroblastoid (sprouter) or epithelioid morphology typical of normal EC monolayers. A normal EC line was less susceptible to modulation. These phenomena were probably caused by a complex interplay of serum and EC-associated growth-modulating substances which is not clearly understood. Several variant forms of ECs have been cloned which retain separate identities.[171]

Schor and Schor[244] found that cultured bovine aortic ECs could be induced to form meshworks of interconnecting sprouters when grown on native collagen gel substrata. Cells, from previously contact-inhibited monolayers, migrated into the gel and formed compact three-dimensional meshworks. This behaviour represented a form of phenotypic flexibility not unlike that of the angiogenic sprouting of ECs in vivo. If monolayers atop gels containing meshing sprouters were digested away, sprouters from the gel would migrate to the surface and regenerate the monolayer. This dual phenotypic potential of ECs was displayed by three clones of bovine aortic ECs used in the study. Sprouters could form on top of or beneath a monolayer depending upon the age of the culture, although the most commonly found sprouter layer was found under the monolayer, on the dish surface. Occasionally, in very old cultures, a

second monolayer would form under the first one and a layer of sprouters was often found to overgrow this.[244]

A variant adult bovine aortic EC line, ABAE-CA1, which produces sprouters has been produced[301] by mutagenic manipulation of the FGF-dependent, monolayering clone of ABAE cells of Gospodarowicz.[114] Of significance in this discussion on sprouters, is the loss by this variant of its contact-inhibition at confluence. It is able to deposit fibronectin on both apical and basal surfaces, unlike ABAE which deposits it basally when monolayered. This adhesion protein seems to mediate attachment and therefore proliferation in three dimensions. One might speculate that the gene complex controlling the phenotypic switching in normal ECs may be permanently locked in sprouting mode in the ABAE-CA1 variants. These variants have lost the ability to make Factor VIII but retain their mitogenic response to FGF.

Growth Factors

The response of subconfluent HUVECs to growth factors present in foetal bovine serum was sufficient to allow serial passage, but was relatively poor when compared to the response of bovine ECs.[179] In addition the clonal growth of ECs, even of bovine origin, was exceedingly slow in media supplemented with FCS (foetal calf serum). These facts stimulated a renewed search for other growth factors which must exist in vivo.

Fibroblast Growth Factor

Abundant reports in the literature,[reviewed 88] in particular those of Gospodarowicz and co-workers, may be found on the effects of fibroblast growth factor (FGF) on the clonal survival, growth rate and cultural morphology or phenotype of bovine aortic ECs.[114] Gospodarowicz purified bovine pituitary FGF[104] and characterised it as a potent mitogen for many cell types[107, 109, 113] including human, bovine and murine fibroblasts;[112] bovine aortic and corneal ECs; HUVECs; bovine luteal cells; bovine, rabbit, porcine,human and guinea pig granulosa cells;[106] and Balb/c 3T3 cells.[111] In addition to its functions as general mitogen for mesenchyme-derived cells and survival agent for ECs at clonal density,[114] FGF was also reported to be necessary to ensure contact-inhibition of bovine aortic and corneal ECs, at the monolayer stage of growth.[105] In the absence of FGF, cells proliferated in a haphazard manner producing multilayered cultures.[283] Contact-inhibited ECs, previously cloned and maintained in the presence of fibroblast growth factor, reverted within three passages of FGF withdrawal to proliferating multilayering cultures expressing fibronectin on all surfaces. These reversions would however not occur until the culture was passaged and thus, once an EC culture had become a contact-inhibited monolayer, FGF could be withdrawn and the contact-inhibited morphology would remain stable for 5 to 7 days postconfluence.[284] Conversely, if FGF was administered to a multilayered culture, never cloned nor maintained with FGF present, it would undergo phenotypic change to the contact-inhibited monolayer described.[283] The stability of a contact-inhibited monolayer in the presence or absence of FGF would appear to be influenced by the presence of an ECM or basement membrane.[108] ECs cultured on ECM in the absence of FGF, but with serum, grew into CI monolayers.

Virtually all the findings described up to this point, relate to Gospodarowicz's ABAE cells which depend on FGF for survival at clonal density, for good proliferation at high split ratio (1:64), and for contact-inhibited monolayering at confluence[115] The mitogenic effect of FGF on many other cultures of bovine aortic ECs at low seeding densities has been firmly established,[88, 70] and has been confirmed with the E3 cells sometimes used in experiments reported in this thesis. FGF has also been found to increase the in vitro replicative lifespan of bovine aortic ECs.[70]

This by no means implies that FGF-independent ECs do not exist. Several reports of FGF-independent contact-inhibited monolayers of ECs have appeared[171, 240, 173, 220] and it is likely

that the line ABAE[114] is an FGF-dependent variant.[88] McAuslan et al.[173] grew vigorous cultures of bovine aortic ECs at clonal density in Medium 199 supplemented with thymidine without FGF. Of particular interest is the foetal bovine aortic EC culture cloned successfully by Rosen et al. without added FGF.[220] A plethora of growth factors and growth-promoting agents, other than FGF, which have effects on EC proliferation have been found.[88] One or a combination of these may explain the sometimes conflicting reports of EC responses to different media, sera and substrata.

ECGF

Maciag et al.[178, 179] isolated a peptide from bovine hypothalamus which was mitogenic for HUVECs at low seeding densities and which prolonged their in vitro lifespan. Although this mitogen, EC growth factor (ECGF, also commercially known as ECGS - EC Growth Supplement) may occur in association with a second FGF-like peptide, in its pure form it was found to be distinct from FGF proper.[178] ECGF is an acid, heat-labile protein of molecular mass 75 000 daltons. Its action in promoting growth in HUVECs at low seeding densities depends on the synergistic actions of a serum supplement and the use of a human fibronectin matrix.[179]

MDGF

Macrophage-conditioned medium promotes the growth of FGF-dependent ECs and substitutes for FGF. The active agent, macrophage-derived growth factor or MDGF , may also promote the growth of ECs in vivo although it has not yet been isolated from serum. MDGF is released by activated monocytes or macrophages which are in evidence at the sites of atherogenesis. The release of MDGF at vascular lesions could stimulate EC growth and contribute to the atherogenic process.[88, 221]

ECDGF

Bovine aortic ECs can synthesize a unique growth factor called EC-derived growth factor or ECDGF which acts on non-endothelial cells but not on ECs themselves.. It is a peptide of molecular mass 20-30 000 daltons, believed to be distinct from PDGF, epidermal growth factor (EGF) and FGF.[89] Gajdusek et al.[89] cultured ECs in medium supplemented with plasma-derived serum, which is free of PDGF and other general cell growth-promoting agents. Smooth muscle cells, fibroblasts and 3T3 cells, which failed to grow in PDS-supplemented media, grew well in the EC-conditioned medium which proved to contain ECDGF.[88]

EGF

Epidermal growth factor (EGF), which is found in serum stimulates the growth of HUVECs but not of bovine aortic ECs which lack EGF receptors.[88]

PDGF

Platelet-derived growth factor, released by aggregating platelets, has little or no effect on ECs which grow equally well in whole blood-derived or plasma-derived serum.[88]

Conditioned Medium

Greenburg et al. found that conditioned medium, removed from a confluent monolayer of bovine aortic ECs, could substitute for FGF in restoring the contact-inhibited monolayered phenotype to multilayered ECs grown without FGF.[117] EC-conditioned medium could also support the growth of ECs seeded at clonal density. It appeared as if a growth factor, or complex of growth factors, produced by ECs themselves was present in conditioned medium. The ability of ECs to "self-condition" their own medium depended directly upon the cell density. Once ECs , seeded at low density into conditioned medium, started to proliferate, the conditioned medium could be withdrawn presumably because the ECs were producing enough of their own growth factors.[88]

Extracellular Matrices and Attachment Factors

Bovine aortic ECs become polar monolayers with extracellular matrices (ECM) composed of collagen, types III and V[238] and IV;[110] fibronectin (FN) and laminin;[110] secreted on their basal aspects.

Thrombospondin, a glycoprotein released from the αgranules of aggregating platelets, is also secreted by cultured ECs into their extracellular matrices.[192] Thrombospondin, like fibronectin, promotes cell adhesion and it may play a role in cementing aggregating platelets together. It follows that it may also be involved (like vWF) in helping platelets stick to exposed subendothelium.

HUVECs[144] and bovine aortic ECs[177] synthesize fibronectin (FN) in vitro and secrete it into the medium and ECM.[23] FN binds strongly to collagen types I-V and proteoglycans.[155] FN is present on the basal surfaces of postconfluent ECs where it mediates contact-inhibition and cell attachment to the substratum upon which ECM is laid down. Subconfluent ECs and multilayering ECs have FN on their apical surfaces.[23]

Identification of Endothelial Cells

Several reliable criteria have emerged for the positive identification of ECs in culture.

1) In descending order of importance, **Weibel-Palade Bodies, polar basement membrane** and **endothelial vesicles** (EVs) are characteristic of ECs. WPBs and basement membranes are unique to ECs but vesicles like EVs (micropinosomes) occur in other cells but less abundantly. Despite an early report,[26] Weibel-Palade bodies are not usually seen in cultured bovine aortic ECs.[245, 61] These are all ultrastructural features detected in ultrathin sections of ECs and have been more fully dealt with in Chapter 1.

2) **Angiotensin-converting enzyme** (ACE) is found on the luminal surfaces of most ECs , but mainly pulmonary and aortic ECs . Its detection by immunocytochemistry serves as a useful but not unique marker for the identification of ECs.[232]

3) The glycoprotein complex, Factor VIII (see Chapter 1) plays a key role in the intrinsic coagulation cascade and also mediates platelet aggregation. Cells in vitro produce a non-procoagulant form of Factor VIII[145] which is now known to be **von Willebrand factor** (vWF).[288] vWF, as detected immunologically, has become known as Factor VIII-related antigen. It is synthesized by ECs and is probably the most widely accepted distinguishing characteristic of these cells in vivo and in vitro.[245] The only other entities reported to have Factor VIII-related antigen associated with them are megacaryocytes[194, 140] and platelets.[140]

Morphology of Vascular Endothelial Cells in Culture

Vascular ECs form a monolayer, the innermost layer of the intima. This is in direct contact with the blood or lymph on the luminal (or blood) front and with the interstitial fluid and basement membrane on the abluminal (or tissue) front. When dissociated from blood vessels and cultured in vitro,

ECs retain this polarity which makes them especially suitable as models of endothelium. One of the major in vivo characteristics (partly diagnostic for ECs) which is usually retained in vitro, is this tendency to form monolayers. When ECs reach confluence, they continue to divide until they reach saturation or stationary density which is of the order of 10^5 cells/cm^2.[67]

Thus contact-inhibition of cell division is expressed some time after confluence has been attained. It is such postconfluent cultures which express the typical endothelial morphology - quiescent monolayers consisting of homogeneous, flat, closely-apposed,epithelioid, polygonal cells creating the effect of a cobblestoned pavement.

Confluent ECs will not respond to serum growth factors by mitosis. They remain quiescent in the face of replenishment of exhausted growth factors with fresh serum.[126] This quiescence would be demanded in vivo for monolayer maintenance in the presence of all manner of hormones and growth factors circulating during during normal physiological processes. The retention of this characteristic in vitro further validates the use of ECs in vivo as models of the endothelium.

Mechanical wounding or physical removal of cells from a quiescent EC monolayer in vitro releases the adjacent cells from contact-inhibition.[286] They migrate into the open wound space and mitosis will occur if they cannot fill the space by spreading out. This regeneration of the monolayer has its exact counterpart in intimal wound healing and re-endothelialization in vivo.[248]

While actively growing or preconfluent, ECs appear elongated or even fusiform: once fully monolayered and contact-inhibited the cells are extremely compact and polygonal. In vivo the mature endothelium consists of elongated ECs, their long axes aligned with the direction of blood flow. Bovine aortic ECs cultured under unidirectional hydrodynamic shear forces also appear elongated along the lines of the force.[69, 73, 65, 141]

Senescence

Cultured ECs display the "Hayflick Phenomenon",[129] having a limited lifespan in vitro and a low frequency of spontaneous transformation.[164] Levine et al.[193, 164] described the general lifespan of foetal bovine aortic ECs as a stationary phase of approximately 60 population doublings, and a decline phase of approximately 20 population doublings. These correspond to the Hayflick phases II and III. During stationary phase, cultures achieved maximum confluent density after each subculture, but during decline the densities dropped off dramatically until cells ceased to grow. The number of population doublings, and not time in culture, appeared to determine the limitation of lifespan. Thus genetic, not metabolic, factors may explain the mortality of cultured ECs. Age-related changes included an increase in average cell size probably due to an increasing population of larger cells, and a decline in the angiotensin-converting enzyme activity . Factor VIII-related antigenicity was retained throughout the lifespan. Mutagenic treatment,[164] thymidine feeding[173] and FGF treatment[70] have all been shown to prolong the in vitro lifespan of ECs.

Interaction of Human LDL with Bovine EC Cultures

Because the broad research field against which this thesis must be seen involves the mechanisms of human atherosclerosis, some justification of the use of a bovine cell culture model is needed. Criticisms have been levelled at the use of non-primate and even non-arterial ECs in atherosclerosis research.[270] Although the need for homologous systems in the study of the cell biology of human diseases is well recognised, human arterial ECs have been notoriously difficult to isolate, maintain and propagate by long-term serial cell culture.

Pending the development of routine methods of culturing human arterial cells, this study was conceived and executed using the best-defined arterial endothelial model then available, bovine aortic ECs .

In 1983 a method for routine culture of human arterial ECs appeared which described successful cloning, rapid proliferation and extended lifespan in heparin and ECGF-supplemented medium.[274] In the same year, Van Hinsbergh et al. reported LDL and AcLDL metabolic studies in cultured human umbilical arterial ECs. ECGF and fibronectin but not heparin were used as cultural aids.[279] In view of the relatively high LDL binding levels it was possible that the confluent arterial EC cultures were not fully quiescent. Contact-inhibition of the confluent arterial ECs was assessed by monitoring thymidine uptake autoradiographically, but the data were not given. Comparison with other systems was thus not possible.

Endothelial Cell Cultures compared with Endothelium

In the foregoing Introduction, EC culture has been reviewed. In Chapter 1, the structure and function of ECs as they occur in the endothelium have been dicussed at some length. There appears to be little advantage in a detailed point-by-point comparison of these properties because the degree of structural and functional congruence between ECs in vivo and in vitro is remarkable. ECs are specialised as boundary cells, growing on a solid, self-secreted basement membrane as a flat sheet with their apical surfaces exposed to a liquid phase. Their adaptation to flat substrata in cell culture with virtual retention of their in vivo phenotype is not surprising in the light of this insight.

There are instances where data obtainable from cultured cells have for technical reasons not been available from cells in vivo, e.g. the expression of ECDGF and ECGF. In these cases comparisons are impossible. Apart from these the only clear differences relate mainly to the results of isolation and selection of ECs which must occur during culture. The effects of this might be selection of variants not representative of the population in vivo; degeneration of cells and their interactions with one another due to accelerated senescence caused by frequent subculturing; and alterations caused by lack of essential growth factors or nutrients.

Sprouting of ECs in culture remains a problem in terms of its explanation. It could be the result of a selection process but is more likely to result from the depletion of some essential inhibitor which is present in fresh serum.[171] The practical problem of sprouting may be circumvented by use of FGF-dependent cells or by selection of variants with a low tendency to sprout (see above).

The loss of typical tight and gap junctional complexes in serially passaged EC cultures is well known and may be the result of senescence. Haudenschild[125] comments on the "leakiness" of endothelial monolayers in vitro and describes the degenerate junctional complexes found in human and bovine ECs in vitro. This only becomes a practical problem in transport experiments where passaged cultures should not be used. Primary cultures retain well-developed junctional complexes[162] and may be suitable although most recent transport studies have been done in situ[280, 57] or in vivo.[257]

In the present study, the main requirement was for clearly-identifiable EC cultures expressing contact-inhibited postconfluent monolayers on well-developed basement membranes. The expression of LDL receptors was a mandatory requirement. All these requirements could theoretically be met by bovine aortic ECs in simple dish culture since no transport experiments were envisaged. Accordingly, foetal bovine ECs were isolated and characterised as described below.

MATERIALS and METHODS

All reagents used were analytical grade (A.R.), unless otherwise stated.

For giving the percentage concentrations of solutions, the following generalisations apply. If liquids or solids were dissolved, their concentrations were given as the volume (ml) or mass (g), respectively, of solute dissolved in 100 ml of the solution, and expressed as a percentage.

Unless otherwise specified, solutions should be assumed to have been aqueous, made up in double glass-distilled water (DGDW).

All cell culture media were pyrogen-free and all cultures free of mycoplasmas.

Cells and Culture Techniques

Bovine Aortic Endothelial Cells

Foetal bovine aortic ECs of three types were used in this study : E3, E7 and E8. E3 (formerly designated A3 C12) was cloned in our laboratory from ECs derived from the thoracic aorta of a 2-3 month old bovine foetus.[60] E7 was isolated in the similar way to E 3 but was not cloned. E3 and E7 were prepared by a modification of the method of Ross[227] as follows.

Bovine foetuses, in utero, were obtained from a local abattoir and the initiation of primary ECs culture commenced within two hours of maternal sacrifice. A foetus was removed aseptically from the uterus and washed thoroughly in iodine-povidone complex (Betadine, Adcock-Ingram Laboratories, Johannesburg), and the heart and aorta excised via a lateral thoracotomy. The aorta was separated from the heart, dissected clean of adherent fat and connective tissue, rinsed in PBS and placed in a petri-dish of fresh PBS. The aorta was slit longitudinally, opened out flat, and then sterile cotton-wool buds were gently swabbed over the intimal surface easily removing the endothelial layer in small sheets. The cut edges of the vessel were carefully avoided in order to reduce the pickup of SMCs or fibroblasts. Adherent cells were swizzled off the cotton-wool bud into medium (MEM) formulated as described below.

Methods based on those of Jaffe[147, 142] were used to isolate line E8, the ECs culture most extensively used in this thesis. E8 was enzymatically dissociated from the intima with collagenase[2, 114, 93] and cultured in the presence of FGF. Up to the separation of the heart from the aorta, the procedure was similar to that used for E3 isolation. The intercostal arteries and other side vessels were tied off with sutures and the open ends of the aorta cannulated with sterile nylon tubes. The lower tube was connected to a stop-cock, and the lumen of the aorta filled with a sterile 1mg/ml solution of collagenase in MgPBS, and incubated for 30 minutes at 20-25^{o}C. Collagenase was Type II (CLSII, Worthington Biochemical Corp., Freehold. New Jersey, USA) which contains high clostripain and tryptic activities. After incubation, the collagenase solution with released ECs was drained into a centrifuge tube containing a small quantity of serum. The drained aorta was slit open and the intima very gently denuded of the remaining ECs with a cotton-wool bud as described above. Cells were transferred to the collagenase solution, washed by centrifugation in MEM-FCS and seeded into a 25cm^2 flask. FGF(BRL, Gaithersburg, Md. USA) was added every 48 hours, to give a final medium concentration of 100 ng/ml. The cells, designated E8, grew rapidly, and were free of contaminating fibroblasts and SMC. At the third passage, parallel cultures, some with and some without FGF supplements, were set up. By passage 11 it was clear by inspection that

growth at split ratios of 1:4 was rapid in all cultures, and that contact-inhibited monolayering was independent of FGF. So the use of FGF was discontinued. After 10 further passages the contact-inhibited monolayering at confluence was not lost. The morphology of E8 cells passaged either by collagenase or trypsin dissociation was compared but no differences in the final monolayer morphology were seen. Collagenase treatment yielded small groups of cells which were difficult to separate whereas trypsin easily dissociated cultures into single cells. Trypsin was therefore used in passaging procedures. No attempt was made to keep track of the number of population doublings.

Human Skin Fibroblasts

Human skin fibroblasts isolated from normolipaemic subjects served as positive controls and standards for the binding and metabolism of LDL in this thesis. These were GM 0203, GM 3348 and FGo. The normal fibroblast designated FGo, was isolated from a normocholesterolaemic Afrikaner subject by Van der Westhuyzen et al.[278] Mutant fibroblasts GM2000 and GM0701, which lack functional LDL receptors and do not bind LDL, served as negative controls.

GM 2408A, designated J.D. after the patient from which it was isolated, is a mutant fibroblast which, while producing receptors capable of binding a normal amount of LDL, is incapable of internalising these.[7] GM 2408A is a compound heterozygote consisting of one R-0 allele, producing a non-functional receptor, and one R-160[1] allele which produces an internalisation-defective receptor. The molecular lesion responsible for the internalisation defect is a point mutation in the DNA region coding for the cytoplasmic domain of the receptor: adenosine is substituted for guanine converting a tyrosine codon to a cysteine codon. After transcription and translation, an aminoacid substitution occurs 33 residues from the carboxyl-terminus of the receptor protein, at position 807, resulting in two cysteine residues instead of the single one at position 818.[101] The cytoplasmic domain of the receptor molecule, which is near the carboxyl-terminus, is the most likely site of clathrin interaction. Since association of receptors with clathrin-coated pits is impaired along with internalisation in GM 2408A, it is likely that the mutated cytoplasmic region of the receptor has defective interaction with clathrin. The precise nature of these defective interactions is unknown.[101] The GM 2408A or J.D. mutant fibroblast strain is one of five known Class 4 mutants with internalisation defects of the LDL receptor. It is noteworthy that, of four such strains subjected to molecular genetic analysis, all had defects in the cytoplasmic domain of their receptors.[101]

Cultures designated GM were purchased from the Human Genetic Mutant Repository (Camden, New Jersey, USA).

Cultivation Methods

All cultures were maintained routinely at 37^{0}C in Eagle's minimal essential medium (MEM) buffered with Earle's salts (Flow Laboratories, Ayrshire, Scotland) supplemented with 10% heat-inactivated bovine foetal calf serum (FCS) (State Vaccine Department, Pinelands, South Africa) and 10% tryptose phosphate broth (Difco Laboratories, Detroit, Michigan, USA) and 60ug/ml benzylpenicillin (Novo Pharmaceuticals, Johannesburg) and 100 ug/ml streptomycin sulphate (Petersen Pharmaceuticals, Epping, Cape Town). Cultures were incubated in an atmosphere of 5% CO2 in air in a humidified CO2 incubator. Where indicated, FGF at 100 ng/ml medium was added every 48 hours. For passaging, cultures were dissociated by treatment with 0.05% trypsin (Difco) + 0.02% EDTA in phosphate buffered saline (PBS) at

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* GM 2408A (J.D.) cells were used as standards for LDL receptor distribution studies by fluorescence microscopy reported in Chapter 3 and those by electron microscopy reported in chapter 4.[3]

37°C for 5 minutes. Cells were passaged at a split ratio of 1:4. Final confluent densities achieved varied from 1.14 to 1.70 x 10^5 cells/cm^2.

Postconfluent and subconfluent cultures representing quiescent monolayers and actively-dividing ECs respectively, were required for experiments. Seeding densities of 10p4 cells/cmp2 yielded postconfluent cultures within 7-10 days. Seeding densities of 7500 cells/cm^2 were used to produce actively-dividing subconfluent cultures after 2-3 days incubation. If fewer cells and longer incubation times were used to produce subconfluent cultures, then patchiness with poorly separated individual cells resulted. Colonies formed whose central cells were confluent: only the peripheral cells were dividing while central cells were quiescent. The performance of receptor-mediated endocytosis of LDL, characteristic of actively-dividing cells, by these latter so-called subconfluent cultures was only marginally better than that of confluent cultures. Seeding at higher densities and growing for a short time produced subconfluent cultures with a higher proportion of active cells as determined by morphological comparison by phase contrast microscopy. These cultures were not sparse, in the sense of having thinly distributed yet active cells, but subconfluent, having a relatively dense distribution of active but still separated cells (see PLATE 2.2).

Growth Rates

To compare the efficacy of gelatin-coated plastic with that of plain plastic as a culture substratum for ECs, growth experiments were undertaken using E3. A 1% sterile solution of bacteriological gelatin (Difco Laboratories, Detroit, Michigan, USA) was poured into the dishes to be coated, the excess removed by suction and the coated surface dried under a UV lamp for several hours. E3 for growth rate experiments were seeded (see Fig. 2.1 below) into 50 mm diameter plastic culture dishes, either plain or pretreated with gelatin. At appropriate intervals during a 14 day growth period, duplicate dishes were trypsin-treated to remove the cells which were counted in a Coulter counter, Model Zf (Coulter Electronics, Hialeah, Florida, USA). Growth rate experiments were conducted over two weeks during which cultural morphology was assessed daily by phase contrast microscopy. The growth rates of E3 cells (seeded at 3500 cells/cm^2) cultured with and without FGF supplements were also measured as described above.

Characterization of Cell Cultures

Endothelial cell identity was checked by the following criteria:

a) Morphology, as determined by light microscopy of living and fixed material.

b) Ultrastructure of confluent monolayers, as determined by transmission electron microscopy.

c) Presence of surface Factor VIII, as determined by indirect immunofluorescence microscopy.

Morphology: Light Microscopy

Phase contrast Microscopy

The cultural morphology of ECs was examined routinely by means of standard phase contrast microscopy with an inverted microscope (Leitz Diavert). Photomicrography was carried out with the aid of a Wild Photo Automat MPS 55.

Silver Nitrate Staining of E C Cultures

Silver nitrate solution will preferentially stain the margins of ECs growing in a confluent monolayer in vivo or in vitro. Although the staining reaction* is not diagnostic for ECs the characteristic cell shapes and arrangement were for many decades the most rigorous criteria available for identifying ECs in vivo and in vitro (see Chapter 1).

The method used was modified from that of Florey and co-workers.[79] Confluent EC monolayers were fixed with glutaraldehyde and osmicated according to the standard procedure (see Processing of Cell cultures for Transmission Electron Microscopy, this chapter). A 0.5% solution of AgNO3 was applied to the cells for 15 minutes after thorough washing with distilled water. The excess AgNO3 was removed and, following a brief distilled water rinse, 1% NaCl was added and left for ten minutes. A heavy precipitate of silver chloride was observed which was washed off with distilled water. The reduction of silver chloride to silver metal was hastened by development for 5 minutes with Kodak D19 diluted 1:10 with water. Cells were washed and examined by light microscopy.

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* SILVER STAIN

Concerning the mechanism of the staining reaction, it is possible that negatively-charged compounds like heparin and other proteoglycans of the glycocalyx bind the silver cations. These are then precipitated by halide anions which are essential for the staining reaction.[79] Precipitated silver halide is readily photoreduced to silver metal and it has been found that simple photographic development will enhance the silver stain reaction.[79] The final staining product is brownish black

Electron Microscopy

Both scanning and transmission electron microscopy were used with a variety of preparative techniques to describe the ultrastructure of ECs .[188] EC identity and morphological modulation were ultrastructurally assessed.

The binding and endocytosis by ECs of ligands, such as lipoproteins, cannot be interpreted fully without ultrastructural investigation to give spatial information. Immunocytochemistry using colloidal gold marking of the LDL receptor was effected on cultured cells which were examined whole in the transmission electron microscope. Gold-labelled LDL binding to receptors was also visualized on whole-mounted cells (see Chapter 4).

The morphology of native, acetylated, DiI-labelled and gold-labelled lipoproteins was checked in the TEM. Negative staining was used to contrast these specimens.

Transmission Electron Microscopy

Fixation and Dehydration

The following standard processing procedure was followed at 20-25^{o}C and pH 7.2-7.4 for all TEM preparations unless otherwise stated. Room temperature fixation was usual because cells subjected to low temperature (4-10^{o}C) aldehyde fixation had altered morphology. Contraction, rounding-up and detachment of cold-shocked cells[235] was thus avoided.

Cells, in situ on their growth substratum, were rinsed free of medium and cellular debris with two brief washes in MgPBS. Where experimental conditions demanded low temperature processing, cultures were prechilled to 4^{o}C for 10 minutes (usually in medium buffered with HEPES, see Medium A. Chapter 3) before washing with cold MgPBS. If magnesium-free PBS was used to wash unfixed cells, they detached from the substratum.

Glutaraldehyde fixation[236] was usual except where immunocytochemical methods precluded its use[296] (see Chapter 3). Glutaraldehyde (EM grade, E. Merck, Darmstadt, West Germany) was purchased as a 25% aqueous solution sealed under nitrogen in glass ampoules. Cells were immersed in a 2.5% working fixative solution (in 0.1M sodium cacodylate buffer, pH 7.4) for one hour and then washed twice with the same buffer for five minutes each time. Cells were post-fixed by immersion in 1% osmium tetroxide (OsO_4) in 0.1M sodium cacodylate buffer for 30 minutes. 1% OsO_4 was prepared just prior to use, by mixing equal volumes of 2% aqueous OsO_4 and 0.2M sodium cacodylate buffer. Two washes in 0.1M cacodylate buffer for two minutes each time preceded dehydration of the cells in a graded series of aqueous ethanolic solutions (50, 70, 96 percent by volume) for 5-10 minutes in each; then three 10 minute immersions in absolute ethanol dried over anhydrous sodium sulphate powder. Tubes were covered with Parafilm (American Can Company, Greenwich, USA) during this last dehydration stage on account of the hygroscopicity of absolute ethanol.

Embedding

Cells were embedded in Epon 812 according to procedures modified from those of Luft.[169] Depending on the type of experiment, cell cultures were either detached from their substratum, usually styrene plastic dishes, prior to embedding, or embedded in situ.[227, 188] Detailed procedures for embedding cells are given below in miniprint.

EMBEDDING DETACHED CELLS

Cells were dislodged from the dishes with a silicone rubber "policeman" or detached by dissolving away the inner surface of the styrene dish just under the cell layer[253, 161] with a solvent. In the latter method, after rapid and complete removal of the last absolute ethanol wash by suction, cells were covered with about 3 mm of propylene oxide. The solvent was gently swirled in the dish whilst observing when the osmium blackened cell layer lifted free of the dissolving styrene substratum. The cell sheet together with propylene oxide was decanted rapidly into a glass tube. The solvent was slightly milky due to dissolved styrene from the dish. The cell sheets sank slowly to the bottom of the tube obviating the need for all but the gentlest centrifugation. The propylene oxide was exchanged three times, after five minute soaks, in order to remove completely all dissolved styrene which might interfere with the polymerization of resin in which the cells were later embedded.

Cells which had been removed mechanically from the dishes were transferred to glass tubes along with a small quantity of absolute ethanol. This was removed after gentle centrifugation (200xg for 1-2 minutes) and replaced with propylene oxide which was left in contact with the cells for 10 minutes. The propylene oxide was exchanged twice after gentle centrifugation.

From this point on all cells, whether removed from the dishes mechanically or by the propylene oxide method, were embedded in epoxy resin following the same method.

Epon 812 epoxy resin was prepared exactly as described by Luft.[169] Cells in open glass or polypropylene tubes were soaked overnight (15-18 hours) in 50% (v/v) Epon dissolved in propylene oxide. This highly volatile solvent completely evaporated leaving the cells infiltrated and surrounded by virtually pure resin. The resin used at this stage did not contain the accelerator DMP-30 and so remained unpolymerised at room temperature. Resin was exchanged twice over a period of 8 hours ; the second resin change contained accelerator. Finally, cells were transferred to resin-filled BEEM capsules and centrifuged through the fresh resin to the apices of the capsules. The resin was heat-polymerized in a temperature gradient from 37-60°C over 24 hours followed by a further 48 hours at 60°C. The hardened resin blocks were trimmed on a Reichert TM 60 Specimen Trimmer (or LKB Pyramitome 11800 pyramidmaker). Ultrathin sections were cut from the blocks on a Reichert Ultracut 4 ultramicrotome using glass or diamond knives. Sections yielding silver to pale straw interference colours (70 - 90 nm thick) were collected on copper grids and stained in the usual way with uranyl acetate[291] for 10-15 minutes, and then lead citrate[213] for 1-5 minutes under carbon dioxide-free conditions. The stains were carefully washed off in jetted distilled water.

CELLS EMBEDDED IN SITU

The in situ embedding methods described below were modified from Ross.[227] Cell cultures were grown in styrene dishes which had been carbon-coated in a Balzers BAE 121 evaporation apparatus and then sterilized by exposure to UV light (325 nm) for 4-8 hours. The fixation and dehydration procedures used were exactly as described above. Cells were infiltrated with Epon 812 immediately after the final absolute ethanol dehydration step. A 50% v/v ethanolic solution of Epon 812 was added to the dishes, still wet with the traces of the last absolute ethanol soak and left for 8 hours. The Epon solution was replaced by pure Epon and after several changes polymerization was initiated in the dishes. A smooth surface cast of the dish, with the cell layer embedded in it, was finally produced. The carbon coating on the dish surface aided in the separation of the resin layer from it. Portions of the casts were re-embedded in resin to facilitate cross-se tioning of the cell layer.

Negative Staining of Lipoproteins

Formvar coated grids were prepared from 0.35% formvar dissolved in chloroform. The lipoproteins, usually diluted to about 0.1 mg/ml in half-strength saline-EDTA (see Chapter 3), were placed onto the filmed surface of a 400 mesh copper grid and allowed to adsorb for five minutes. Excess liquid was removed using filter paper and 10µl of 2% neutral phosphotungstic acid (PTA) applied. The PTA was centrifuged at 8000xg for 5 minutes before use to remove precipitates. The excess was drawn off as above and the grid allowed to air-dry before examination in the TEM at 80 kV

Scanning Electron Microscopy

Introduction

Early morpologists working on the vascular endothelium used celloidin replicas in the TEM to see the intimal surface at the ultrastructural level.[1] In the early 1970's, the development of techniques for routine EC culture and for scanning electron microscopy of cell cultures coincided. Consequently, scanning electron microscopy superseded replication as the method of choice for surface studies of critical point-dried cells and endothelia.[28]

Scanning electron microscopy of cultured ECs provides a relatively rapid and simple means of assessing topographical morphology at a routine resolution of 10 nm or better. The intercellular margins can be visualized, as can the shapes of cells and the overall integrity of the monolayers. Nevertheless, a case has been made by Schwartz[249] for the use of surface replicas for higher resolution study of EC topography by transmission electron microscopy. Fine surface details, such as caveolae and cell junctional overlaps, are lost in the coating procedures for scanning electron microscopy. Replica techniques are discussed more fully in Chapter 1.

Soft biological specimens may shrink to less than 50% of their original wet fixed volumes during critical point drying (CPD).[27] These shrinkage problems apply to cell cultures prepared for scanning electron microscopy and also to replicas prepared according to Schwartz,[249] which also involves critical point drying. Boyde quantified the relative degrees of shrinkage at all stages of the processing of mouse embryos for scanning electron microscopy and included a comparison of two drying methods, critical point and freeze drying.[27] Critical point-dried embryos underwent 24% reductions in linear dimensions (relative to fixed wet material), while freeze-dried embryos only shrank by 4.6%. Clearly the volume change implicit in a 24% linear shrinkage is gross and although not readily measurable in monolayered cells, will cause severe damage. Boyde demonstrated this point by presenting evidence of intercellular cleavage or separation of cells in a monolayer of rat endocranial osteoclasts.[27] He advocated the use of freeze-drying with cell cultures.[28]

In the present study, confluent monolayers of bovine aortic ECs were severely torn after critical point drying. Tearing was much less apparent in specimens of subconfluent cells which had not elaborated a basement membrane. In this case, separate cells were able to shrink without exerting any tension on their neighbours. Conversely, interconnected confluent cells, firmly attached to the substrate, exerted strong tearing forces on one another as they shrank causing tears to develop at the weakest points.

Tearing has been reported in critical point dried endothelium in situ. Davies et al.,[66] after examining rabbit endothelium in situ by scanning electron microscopy concluded that airdrying eliminated tearing artefacts resulting from lipid extraction by organic solvents used in the dehydration steps prior to critical point drying.[103] In discussing the mechanism of the severe shrinkage during CPD Boyde[27] suggests that CPD per se does not cause shrinkage, but that incomplete removal of organic solvents used for dehydration and intermediate fluids may be responsible. In accordance with the above findings, freeze-drying techniques were used for SEM preparations of confluent ECs.

Freeze-Drying of Cultured Cells for Scanning Electron Microscopy

Confluent ECs were grown in untreated plastic dishes or on dishes which had been pre-treated with a 1% gelatin solution and dried under UV light overnight. Initially, experiments were performed on glass coverslips, but since confluent cell layers detached rather easily, especially during the freeze-drying steps, this practice was abandoned in favour of plastic dishes.

The over-all method has been modified from that of Boyde.[28] Processing was as described previously for embedded material except that post-fixation was done in 0.5% osmium tetroxide

for 15 minutes which was found empirically to give the best surface preservation. After osmication cells were washed twice, 5 minutes each time, in PBS and then twice in chloroform-saturated double glass-distilled water.[28] Pieces of plastic dish with adherent wet cells were removed from dishes with a hole-saw, and plunged into stirred liquid propane cooled by liquid nitrogen. This method of plunge-freezing, based on the methods of Robards,[214] can give a very rapid cooling rate (20 000^{o}C/second) resulting in minimal specimen damage by ice crystals. The plastic pieces were removed quickly from the freezing bath (excess propane flicked off) and held under liquid nitrogen during transfer to the freeze-drying chamber. This was the chamber of a standard Balzers high vacuum coating unit BAE 121 which contained a specially modified freeze-fracture stage to which the flat plastic pieces could be attached rapidly with spring clips. The stage was held at -100^{o}C by means of a liquid nitrogen pumping system controlled by a Balzers freeze-etching control unit GA-1. Freeze-drying took place in a vacuum of the order of 10^{-6} Torr over a period of approximately one hour. When all ice had sublimed off, the stage was allowed to reach ambient temperature, and the specimen removed and coated as described below.

Critical Point Drying of Cell Cultures

Cells for critical point drying were usually cultured on glass 8 x 22 mm coverslips. They were processed as described for freeze-dried material up to the dehydration stage in absolute ethanol. Cells were immersed in the intermediate fluid, dried absolute ethanol, in the chamber of a Balzers CPD apparatus and critical point dried. The ethanol was replaced by purging the chamber with liquid carbon dioxide three times for two minutes with 30 minute intervals between purges. This removed all traces of ethanol from the chamber and allowed time for solvent exchange to occur across cell membranes (permeabilized by osmium) so that cells were permeated with carbon dioxide . The chamber was then sealed and the temperature taken up to 35^{o}C, above the critical temperature for CO2 (31.1^{o}C). Concomitantly the pressure rose well above critical (1072 bar) and the phase change occurred from liquid to gas without any surface effects. which damage the surface ultrastructure of specimens. The pressure was lowered gradually over a period of 15-20 minutes whilst the temperature was kept constant at 35^{o}C. Once the chamber reached ambient pressure the specimens were stored in a vacuum desiccator before coating.

Coating of Specimens

Specimens were coated with spectroscopically pure carbon evaporated from an electric arc housed in a shuttered apparatus within a Balzers BAE 121 coating plant. Specimens were rotated in a special rotary-coating attachment which ensured uniform yet thin carbon layer deposition during evaporation.[295] Voltages of 8 and 16, at 5-8 Amps were used routinely. After the carbon, a thin layer of gold-palladium (60:40) was evaporated from a tantalum boat onto the rotating specimens.

Scanning Electron Microscopy

Specimens were examined in Cambridge S180 SEM at 25kV at 45^{o}. To increase contrast of images of flat specimens such as confluent EC monolayers, the detector signal was differentiated and combined with undifferentiated signal in various proportions until a satisfactory image was obtained.

Factor VIII-Related Antigenicity: Indirect Immunofluoresce Microscopy

The procedures described below were modified from the methods of Schwartz.[77] E3 and As1C8 bovine SMCs (the negative control) were seeded ($2x10^4$ and $5x10^4$ cells/cm^2 respectively onto glass coverslips) and grown to confluence. They were rinsed in phosphate buffered saline containing 2mM Mg^{2+} (MgPBS). Coverslips of cells were fixed in cold 100% acetone for 10 minutes, equilibrated£ to room temperature in acetone, air-dried and framed for immunocytochemistry.

An antiserum raised in rabbits to human Factor VIII-related antigen (Factor VIII-R Ag) (Clotimmun, AHG-associated protein, OTOM 05) was purchased from Behringwerke (Marburg, West Germany). This rabbit antihuman Factor VIIIRAg antiserum (RAHFVIII), the primary antiserum, was diluted 1:5 with MgPBS and placed on appropriate cell areas on coverslips which were incubated for 30 minutes at 37°C in a humidity chamber. Four ten minute MgPBS washes at 20-25°C were followed by a further 30 minute incubation under the same conditions but with a secondary fluorescent antibody diluted 1:3 with MgPBS and centrifuged before use. This was GAR-FITC (Behring, OKTF), an antiserum raised in goats against rabbit IgG and conjugated with fluorescein isothiocyanate (FITC).

After washing as described above, coverslips were mounted cell side down in a tiny drop of acetate buffered glycerol (pH 8-9 optimal for FITC fluorescence) on microscope slides. The refractive index of glycerol was so similar to that of the cells themselves that they were poorly resolved with respect to the surrounding medium (glycerol) by phase contrast microscopy. Thus where phase contrast and fluorescence microscopy on the same field were required, cells were mounted in MgPBS instead of glycerol.

For controls either plain MgPBS or normal rabbit serum was substituted for the primary antibody (RAHFVIII).

Preparations were examined for FITC fluorescence using a Leitz Laborlux 12 epifluorescence microscope fitted with a Zeiss HBO 50 lamp. The FITC optics were incorporated into the microscope as a filter block (Ploemopak) consisting of a BP 450-490 nm exciter filter, a dichroic beam splitter mirror passing 510 nm to the ocular and an LP 515 barrier filter.

£ Droplets of glycerine were applied to the coverslips on the cell side covering 4 circular areas each 8 mm in diameter. The entire cell side of the coverslip was aerosol spray-coated with polytetrafluorethylene (PTFE). This coating adhered firmly to the dry areas unprotected by glycerine, but floated on top of the glycerine protected areas, from which it was removed along with the glycerine by soaking in MgPBS for 5 minutes. Four circular areas were now available for immunocytochemical treatment. The hydrophobic PTFE "frame" effectively prevented cross-contamination of adjacent test areas and ensured the economical use of expensive immunochemicals.

Autoradiography

Light microscopic autoradiography of tritiated thymidine uptake by the nuclei of EC cultures was used to assess the degree of contact-inhibition of confluent cultures. Confluent cultures were incubated for 18 hours in medium containing |methyl^{3}H|-thymidine (Radiochemical Centre, Amersham, U.K.) at 2.0μCi/ml in 35mm dishes.[93] After labelling, the cultures were rinsed four times with PBS (to remove unbound radioactive thymidine), fixed by immersion in methanol for five minutes, extracted with 5% aqueous TCA for 10 minutes at 4^{0}C (to remove TCA-soluble radioactivity), rinsed briefly twice with distilled water and air-dried.

Autoradiograms were prepared by the method of Caro.[48] Briefly, 10g of Ilford K.5 emulsion was dissolved in 10 ml of DGDW at 45^{0}C. The resulting emulsion was poured into dishes of methanol-fixed cells covering them completely. The excess was drained off; the dishes air-dried; transferred to light-tight plastic boxes containing fresh dry silica gel desiccant; sealed and stored at 4^{0}C for exposure. After 7 - 10 days, autoradiograms were developed in Kodak D 19 at 17^{0}C for 4 minutes; fixed for 5 minutes at 22^{0}C in non-hardening fixer (240g sodium thiosulphate; 10g anhydrous sodium sulphite; 25g sodium bisulphite; dissolved into 1 litre DGDW) and washed for 20 minutes in DGDW. Dishes were then air-dried and examined directly by phase-contrast microscopy which plainly resolved the nuclei and silver halide grains. Staining was therefore unnecessary.

For each specimen, ten random microscopic fields were recorded on micrographs. These were enlarged and analysed by counting separately the numbers of labelled and unlabelled nuclei. Labelled nuclei had dense deposits of silver grains superimposed upon them. The thymidine index (ratio of labelled to total number of nuclei) was calculated and expressed as a percentage. An index of 5% or less was taken as indicative of a contact-inhibited monolayer. This mitotic index (or lower) was characteristic of ECs which demonstrated postconfluent contact-inhibited monolayering (see Results). It was similar to those obtained by others in studies on bovine aortic ECs[67] (approximately 5%) and HUVECs[93] (approximately 4%).

RESULTS and DISCUSSION

Bovine Aortic EC Cultural Morphology

Phase Contrast Microscopy

Confluent EC cultures were examined by phase contrast microscopy for identification by morphological criteria. Endothelial cell cultural morphology was expressed by E3, E7 and E8: E7 and E8 in culture formed contact-inhibited monolayers of flat homogeneous, closely-apposed, polygonal, epithelioid cell sheets resembling a "cobblestone" pavement (PLATE 2.1); nuclei were centrally located and large. E3 behaved like E7 and E8 but at passage numbers above 15 it began to proliferate after confluence, producing "sprouters"[61] (PLATE 2.3) or multilayering.

In this study, most EC monolayers were stable for about two weeks after the 4-7 days taken to achieve postconfluent contact-inhibition. Typical postconfluent and subconfluent cultures are depicted in PLATE 2.1 and PLATE 2.2 respectively.

The duration of the initial growth period depended on the passage number and seeding density of the culture. After 4 - 6 weeks, cells became granulated and vacuolated, and started lifting off the dishes. Areas of substratum became exposed in this way and, if medium was regularly exchanged, secondary regrowth or regeneration was observed in these areas. The incidence of giant cells increased (PLATE 2.4) and sprouters became more common in cultures which had been passaged often (E3) (PLATE 2.3), and some cultures became totally overgrown[88] even forming multilayers. In senescent cultures, the monolayered cells were observed to retract from one another exposing small gaps between the postconfluent cells. E7 displayed this behaviour even at low passage number and therefore was not used for experiments requiring monolayers with tightly-apposed cells.

PLATES 2.1 to 2.4 show some stages of development of bovine aortic EC cultures from seeding to senescence.

Silver Stain

The intercellular margins of the postconfluent culture were sharply delineated by deposition of metallic silver in the junctional clefts. The characteristic pattern of closely apposed, polygonal, monolayered cells was seen (PLATE 2.5)

Plates 2.1 and 2.2

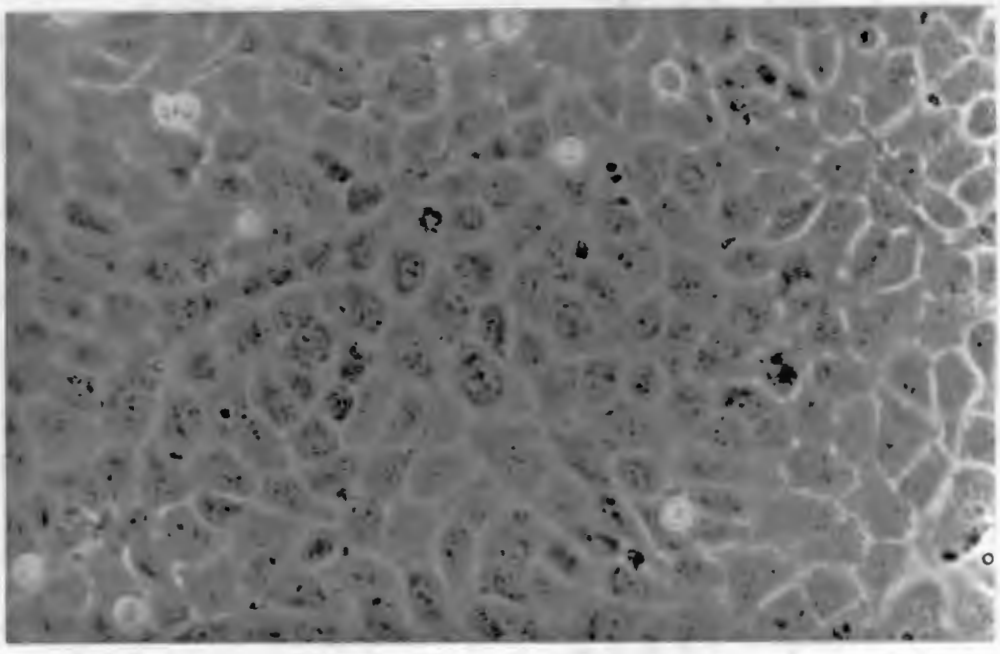


PLATE 2.1: Postconfluent, contact-inhibited monolayer of E8 cells in culture. Note the tightly-packed polygonal cells with prominent central nuclei. The highly refractile bodies are floating cells which have become detached from the substratum. "Cobblestone" monolayers like this were used for biochemical and morphological experiments on high-affinity receptor-mediated endocytosis of lipoproteins (Chapters 3 and 4). Phase contrast micrographs. Magnification 320x.

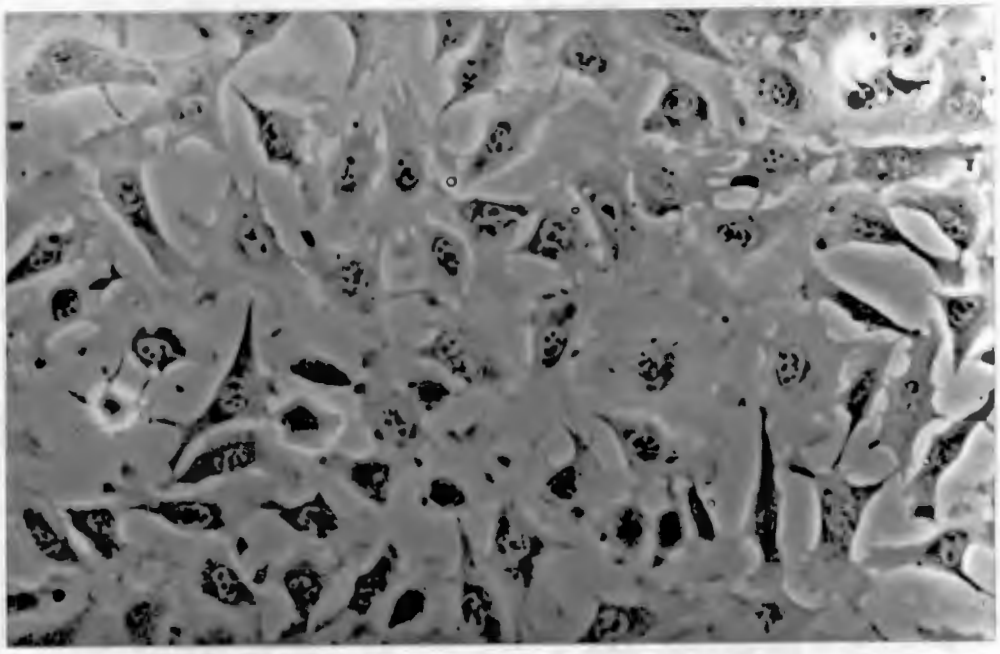


PLATE 2.2: Subconfluent actively-dividing E8 cells were seeded at 7500 cell/cm^2 and incubated for 3 days (the last two in MEM-LPDS). Note the high density of active cells with spreading marginal extensions. Subconfluent cultures like this were compared with postconfluent cultures for receptor-mediated endocytosis of lipoproteins (Chapters 3 and 4). Phase contrast. Magnification = 320x.

Plates 2.3 and 2.4

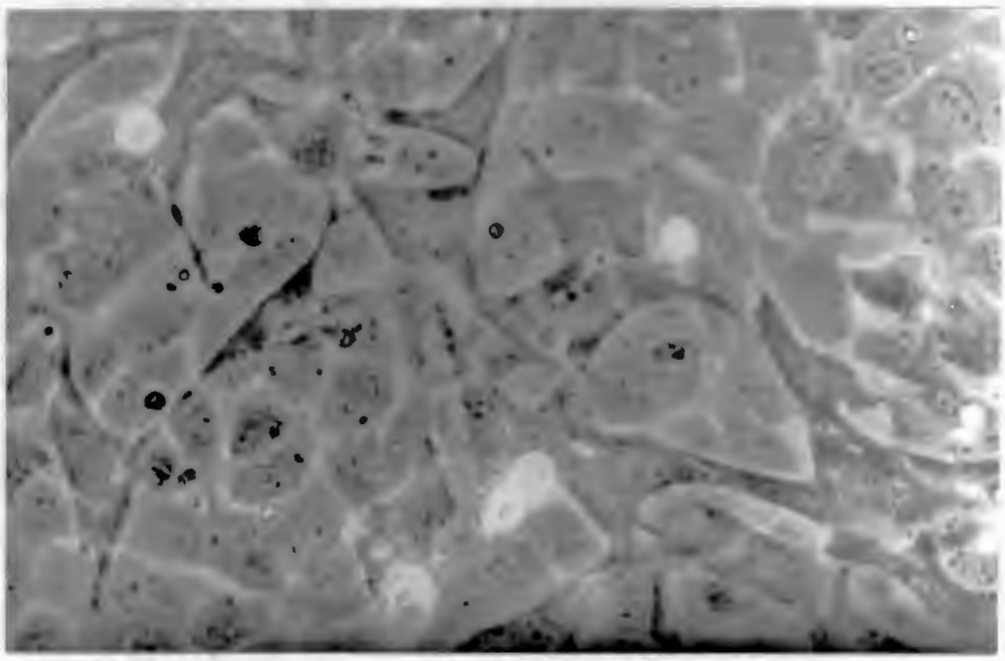


PLATE 2.3: Sprouters in postconfluent culture of E3 cells. The fibroblastoid morphology of the sprouters contrasts with the epithelioid morphology of the monolayer. By gradual progression from under-focus to over-focus with the light microscope, it was possible to localise the sprouters between the monolayer and the dish surface - i.e. it was an undergrowth. Phase contrast. Magnification = 448x.

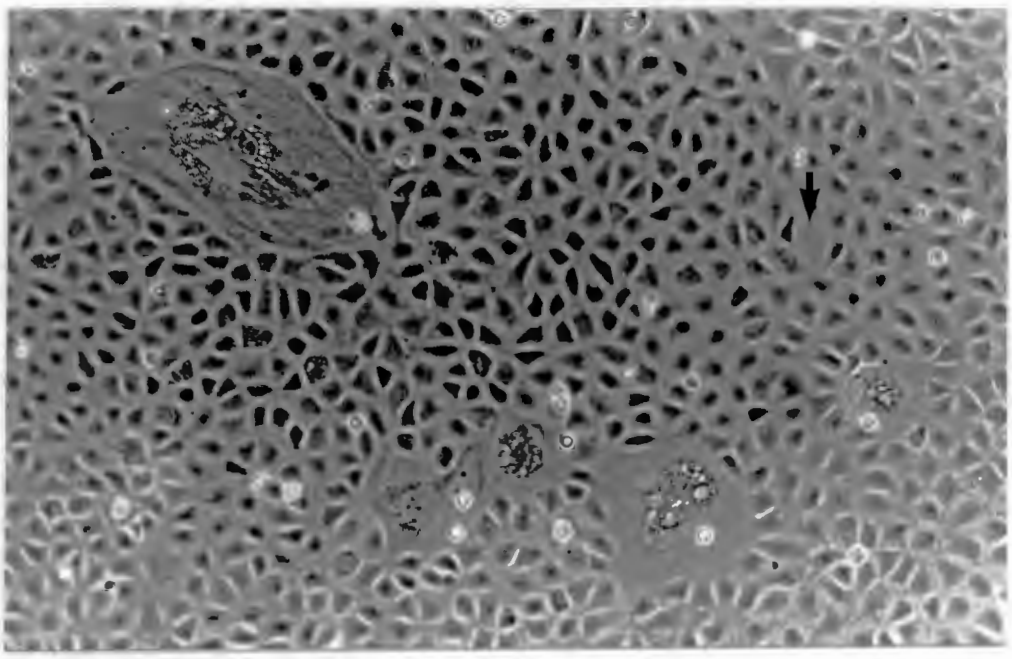


PLATE 2.4: Giant cells in senescent (21st passage) postconfluent E8 cell culture. At least 5 enlarged cells with large central vesicles are visible here). Detached cells (refractile bodies) are evident. A small patch of substratum from which cells have been lost is also visible (arrow). Phase contrast. Magnification = 152x.

Plate 2.5

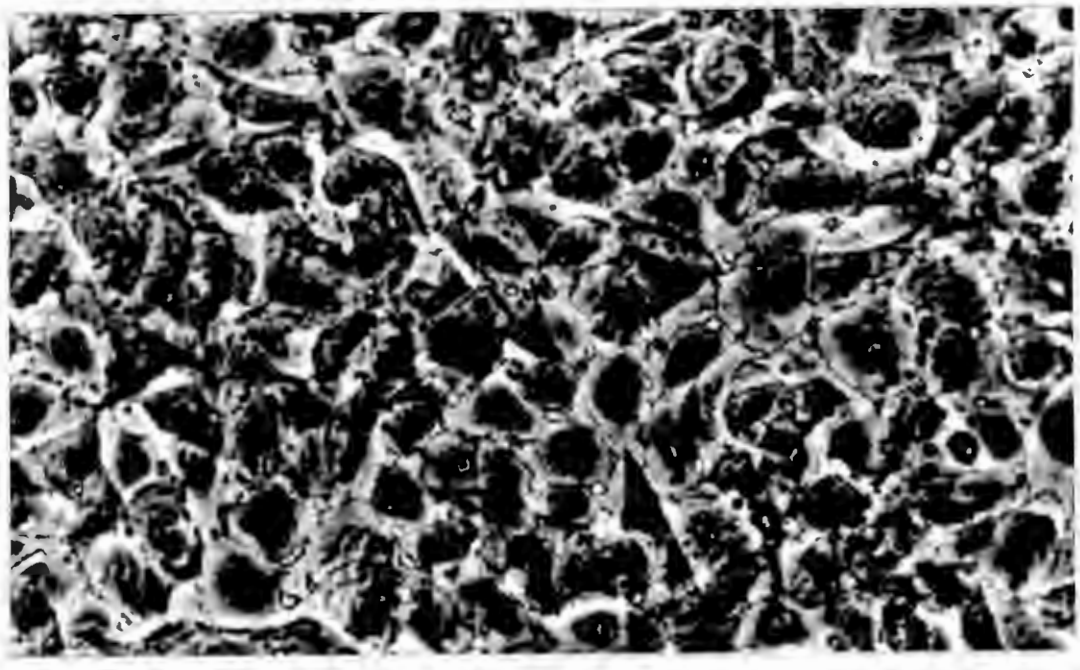


PLATE 2.5: Postconfluent E3 contact-inhibited monolayer stained with silver nitrate and imaged by phase contrast light microscopy. Deposition of silver metal clearly delineates the intercellular boundaries as thin black lines. The dark central areas of each cell are due to the bulging profiles of the cells. Small holes, imperfections in the integrity of the monolayer, show up very clearly. Some of these are probably due to the preparation technique. Magnification = 512x.

Factor VIII-Related Antigenicity

Detection of Factor VIII-R Ag by Indirect Immunofluorescence

Many, but not all, cells in the confluent monolayers expressed the Factor VIII-R Ag which was detected by brilliant punctate fluorescent foci only in preparations treated with a specific primary antiserum. The foci were uniformly and densely distributed over the entire central cell area but were more sparse on the cytoplasmic periphery (PLATE 2.6 A) The fluorescence marked antigens on the surface as the plasma membranes were impermeable to antibodies. Where a non-specific primary homologous antiserum (NRS) was used, diffuse fluorescence, attributable to non-specific binding of the secondary antibody (GAR-FITC), was seen (PLATE 2.6 B).

A cloned bovine aortic SMC line As_1Cl_8, (gift from G A Coetzee) was used as a negative cell control. No Factor VIII-RAg-specific fluorescence, only diffuse fluorescence attributable to the binding of the non-specific secondary antibody (GAR-FITC) was detected on cells incubated with RAHVIII and GAR-FITC (PLATE 2.6 D).

In SMC control preparations in which the specific primary antiserum was substituted by a non-specific homologous (NRS) serum (PLATE 2.6 E), only non-specific, diffuse fluorescence similar to that seen as background to the specifically-labelled preparations, was seen.

In E 3 and As1C8 controls where no primary serum of any kind was used and only GAR-FITC applied, the background fluorescence attributable to non-specific binding of the secondary antibody was negligible (PLATES 2.6 C and F respectively).

Confluent E8 monolayers were subjected to an identical immunofluorescence test for Factor VIII-R Ag and were found to express the antigen.

The antiserum RAHFVIII, is directed against epitopes of human Factor VIII-R Ag or vWF. vWF is also synthesized by bovine aortic ECs in culture, and this bovine vWF has been found to aggregate human platelets.[281] E3 and E8 ECs when incubated with RAHFVIII antiserum and a fluorescent secondary antibody were labelled with large fluorescent foci. Control SMC were not labelled. Because the RAHFVIII is known to cross-react with bovine vWF because of its virtual identity with human vWF[77] it was concluded that E3 and E8 expressed this antigen. This result positively identified these cells as ECs .

Plate 2.6 A-C

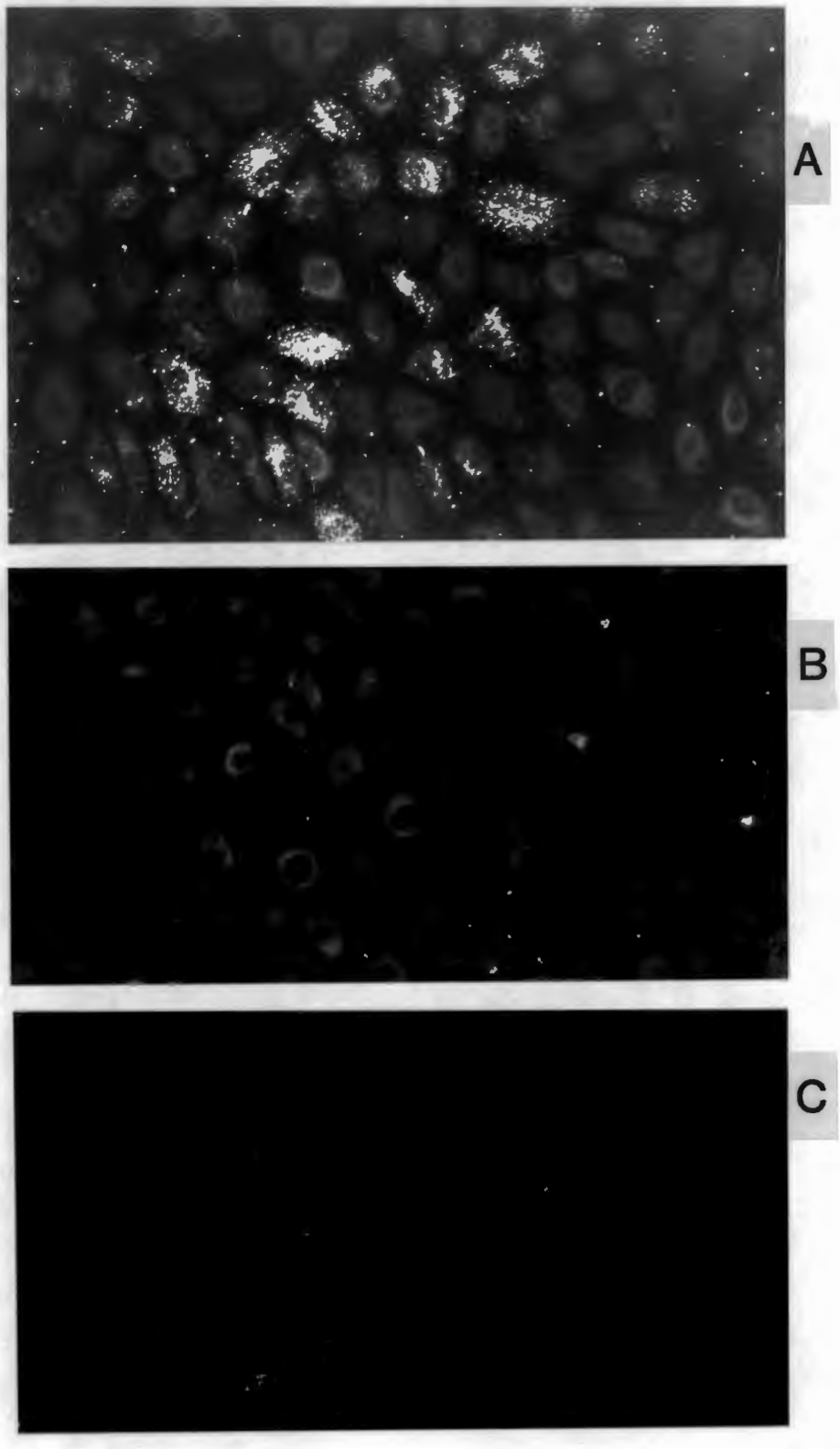


PLATE 2.6 A-C: Confluent E3 cells incubated with **A:** rabbit anti-human Factor VIII:R Ag antibodies (RAHFVIII); **B:** normal rabbit serum (NRS); or **C:** saline: all followed by goat anti-rabbit IgG conjugated to fluorescein isothiocyanate (GAR-FITC). Epifluorescence micrographs. Magnifications (A-C) = 300x.

Plate 2.6 D-F

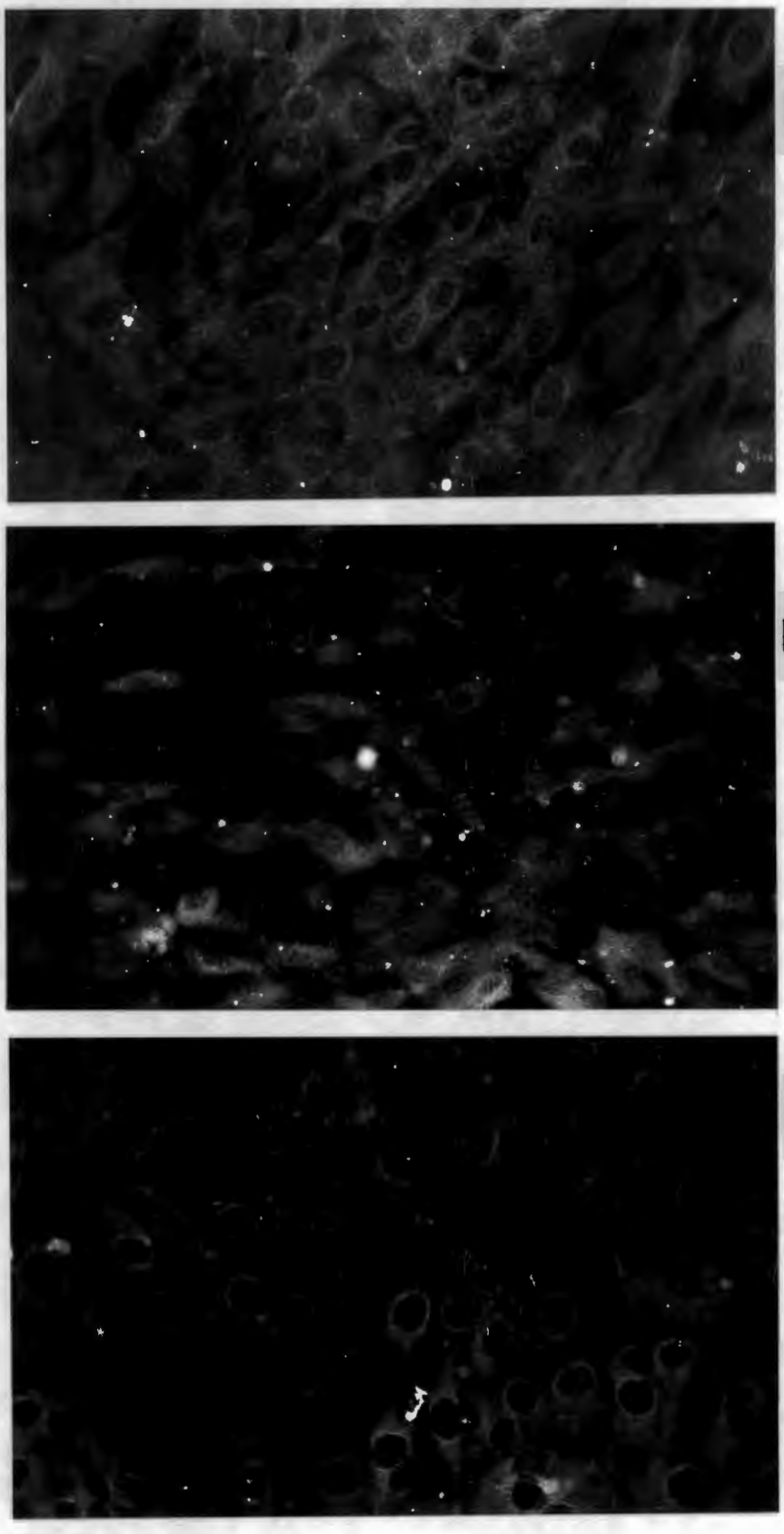


PLATE 2.6 D-F: Confluent AS_1Cl_8 cells incubated with **D**: RAHFVIII; or **E**: NRS; or **F**: saline: all followed by GAR-FITC. Epifluorescence micrographs. Magnifications (D-F) = 300x.

Electron Microscopy

Transmission

When thin sections of confluent EC cultures were examined by transmission electron microscopy, the ultrastructure of EC monolayers revealed cells with bulging apical profiles; abundant endothelial vesicles and caveolae in the peripheral cytoplasm; coated vesicles and pits; lysosomes; rough endoplasmic reticulum; intermediate filaments and microfilaments; long cell overlaps with junctional complexes; and a basement membrane secreted on the basal aspect of the cell sheet (PLATES 2.7 A, B.)

Junctional specialisations were degenerate in the multiply-passaged cells used in this work. While junctional complexes were seen (PLATE 2.8 A-C) no clear tight or gap junctions could not be resolved. The complexes had cytoplasmic densities and are thought to be degenerate.[125] Larson and Sheridan[162] described both gap and tight junctions in primary cultures of bovine aortic ECs. These specialisations, however, became less frequent with repeated passaging. Haudenschild[125] noted that true gap and tightjunctions were less frequent and less well-developed in ECs in vitro than in vivo. He noted that this might cause a degree of "leakiness" in cultured EC monolayers used in transport studies. These deficiencies had no bearing on the study of LDL receptors on cultured ECs which formed the subject of this thesis.

Confluent E8 cultures sometimes produced complex interdigitating intermediate junctional overlaps between cells of the monolayer (PLATE 2.8 D). Taken together, the ultrastructural characteristics of E8 and E3 cells in culture were typically endothelial. No evidence of aberrant or contaminating cells was detected by transmission electron microscopy.

Plate 2.7 A

PLATE 2.7 A: Transmission electron micrograph of part of an E8 cell growing in a postconfluent monolayer on the surface of a plastic culture dish.
A prominent basement membrane (B) is located only on the flattened basal surface (abluminal) of the cell. This polar basement membrane is an extracellular matrix (ECM) containing fibrillar proteins. The apical (luminal) cell surface bulges out into the space which was filled by medium and has microvilli (large arrow) and cytoplasmic extensions (C). An abundance of endothelial vesicles (arrows) populate the cytoplasmic sides of both apical and basal surfaces. These endothelial vesicles are easily distinguishable from the fuzzy coated "vesicles" (arrowheads) which occur less frequently on both surfaces: these "vesicles" may in fact be pit profiles which do not include neck regions (see Chapter 1). A coated pit (open arrow) is clearly visible at the base of a cytoplasmic extension on the apical surface. The large, central nucleus (Nu) is surrounded by bundles of intermediate filaments (i) and mitochondria (m). Rough endoplasmic reticulum (r) is distributed throughout the cytoplasm and occasional lysosomes (l) are seen. Magnification = 15 000x.

Plate 2.7 B

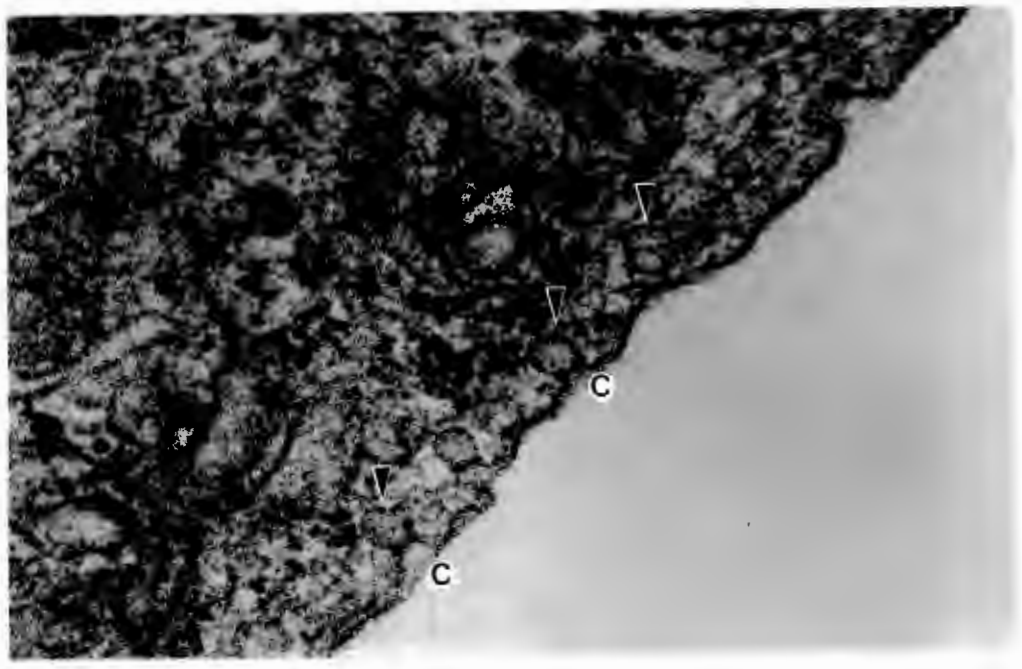
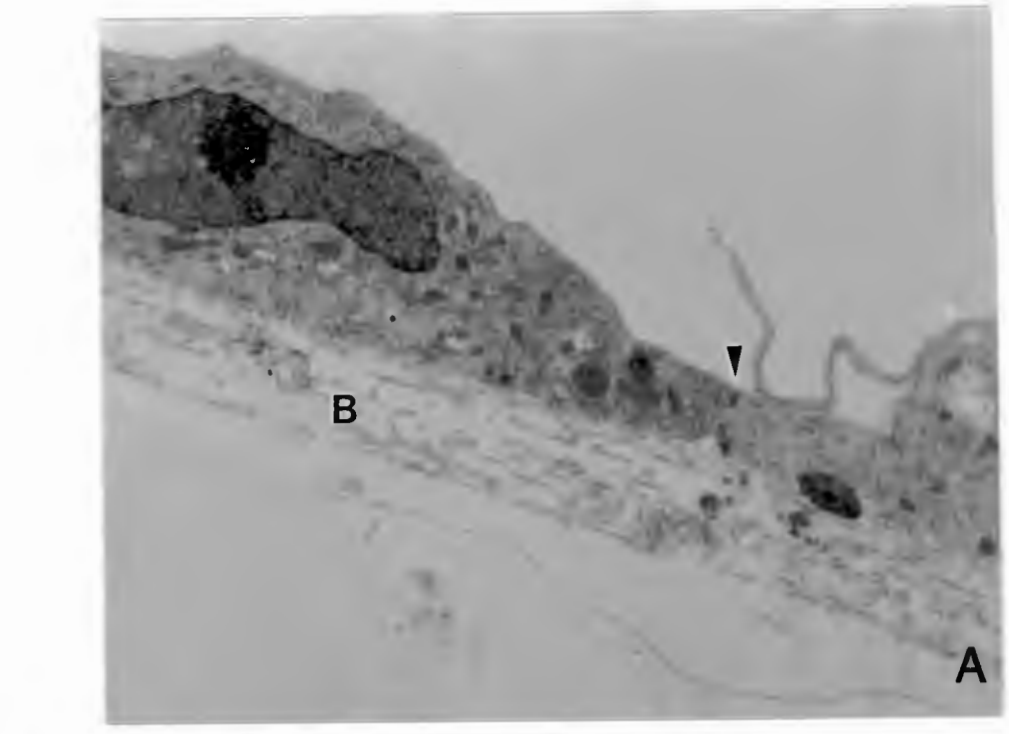


PLATE 2.7 B: Transmission electron micrograph of the luminal front of an E8 cell from a postconfluent monolayer. Endothelial vesicles (arrow heads), apparently free in the cytoplasm and communicating with the extracellular space via necks with apparent diaphragms, are visible. The open vesicles, caveolae (c), look like small flasks. Magnification = 96 000x.

Plates 2.8 A, B



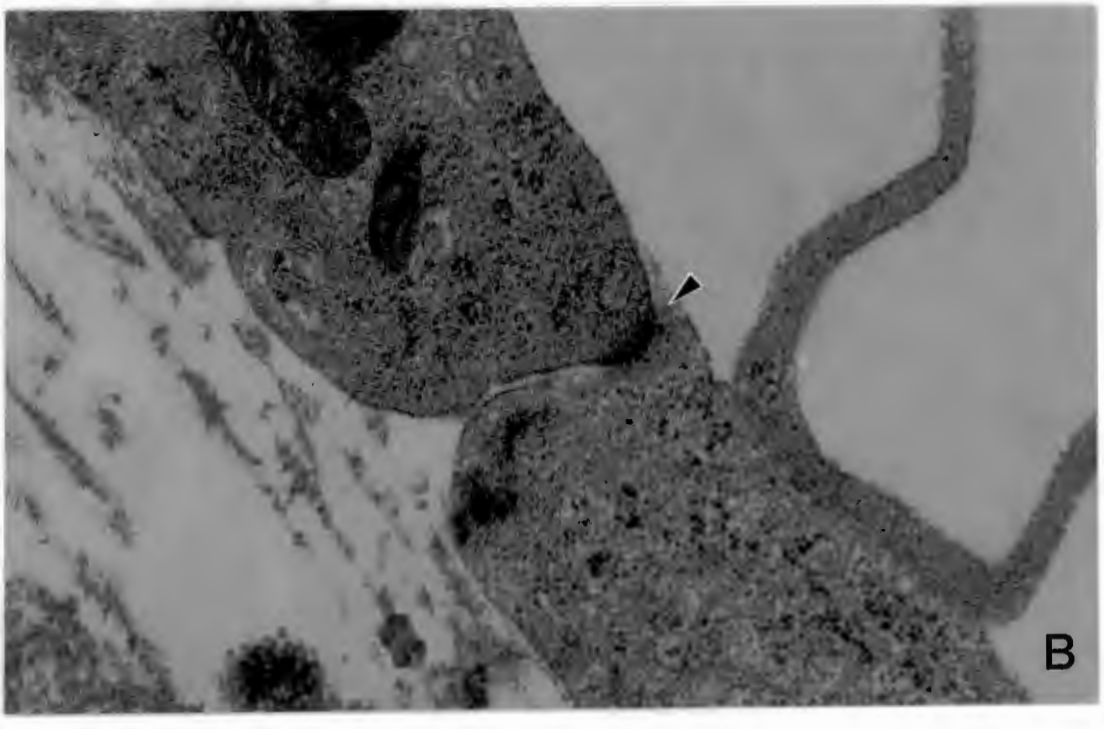


PLATE 2.8 A, B: Low (12 000x) **(A)** and high (60 000x) **(B)** magnification transmission electron micrographs of postconfluent monolayers of E8 cells in culture on plastic surfaces. **A**: The intercellular cleft region (arrowhead) has been imaged and shows the relationship of the cells to one another and the basement membrane (B). **B**: The cleft region detailed (from A) contains an endothelial vesicle open to the intercellular space, and a junctional complex (arrowhead) at the apical end of the cleft. Neither the typical pentalaminar gap junction nor the membrane fusion of the tight junction was seen in this or any other micrographs of the passaged ECs used.

Plates 2.8 C, D

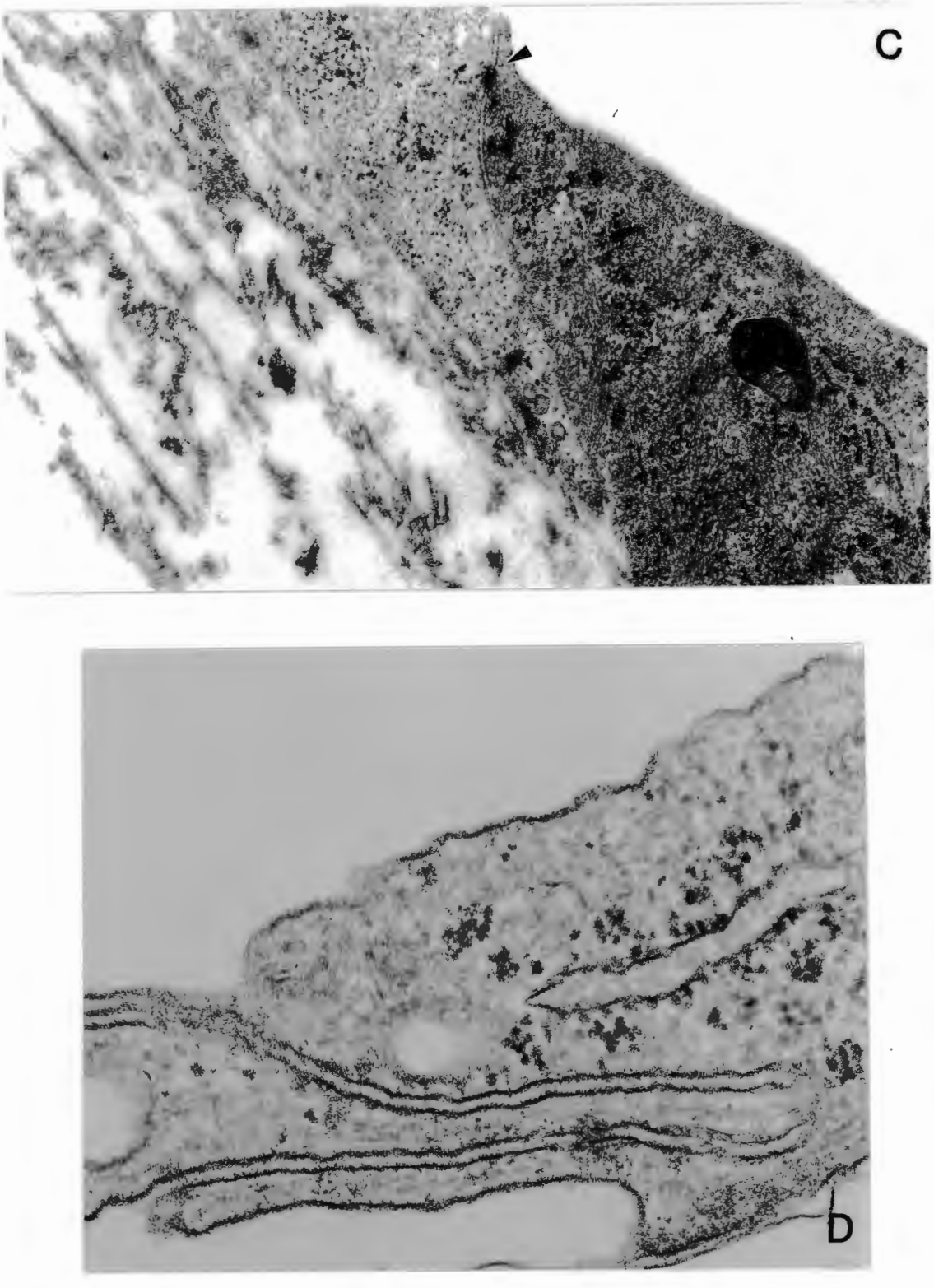


PLATE 2.8 C,D : Transmission electron micrographs of a postconfluent monolayer of E8 cells cultured on a plastic surface. **C**: The cell overlap seen contains an apical junctional complex (arrowhead) near the apical end of the cleft, and very clear parallel membranes forming an overlapping junction. Magnification = 50 000x. **D**: Interdigitating overlaps between E8 cells in confluent monolayer. Magnification = 98 000x.

Scanning

Scanning electron Microscopy of freeze-dried, cultured EC monolayers revealed topographical detail and cultural morphological detail far beyond that available by phase contrast microscopy. The essentially smooth surfaces (with occasional blebs and microvilli) approximated the inner surface of the aortic intima. But the cells were not elongated as they are in vivo by the influence of blood flow (PLATE 2.9 A). Higher magnification scanning images (PLATE 2.9 B) revealed junctions of partially overlapping cell margins. The margins were scalloped and sometimes had microprocesses extending a relatively short distance across the surface of the adjacent cell. Prominent nuclear bulges on the apical surfaces of the cells were generally noted (PLATE 2.9 A, B). Small holes between cells were sometimes detected through which fibrillar basement membrane material could be seen clearly (PLATE 2.9 C). The basement membrane was better accessed in some specimens from which cells had been lost, thus exposing the fibrils (PLATE 2.9 D).

Plate 2.9 A, B

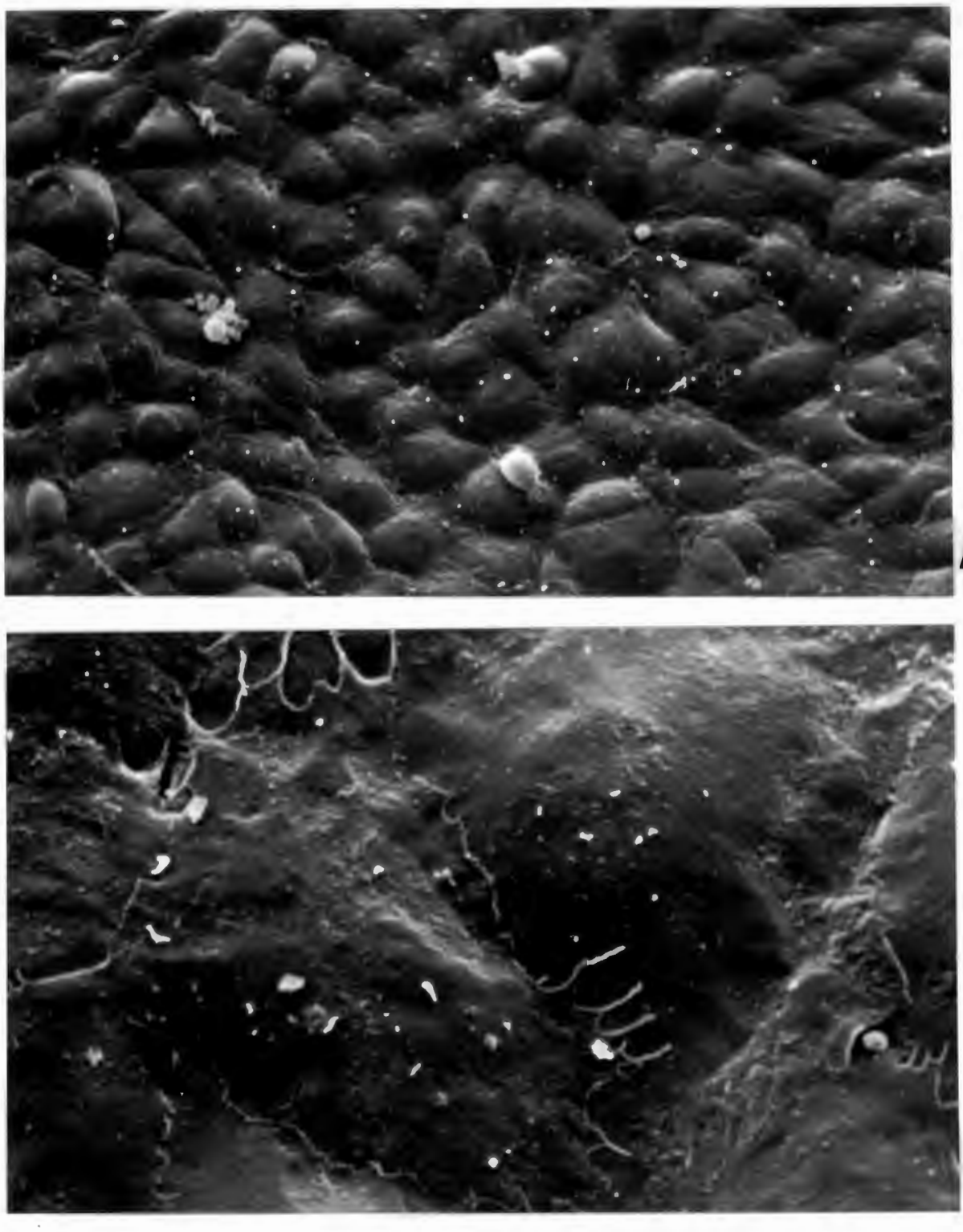


PLATE 2.9 A,B: Scanning electron micrographs of postconfluent E3 cells growing as contact-inhibited monolayers on plastic surfaces. Cells were fixed with glutaraldehyde and osmium; freeze-dried at -100°C; coated with carbon and gold/palladium and imaged at 25 kV.
A: Low power micrograph. Note the absence of tears and the good preservation of the monolayer over a relatively large area. Magnification = 600x.
B: High power SEM image of the intercellular margins of ECs . The scalloped margins appear to overlap slightly. Magnification = 3 700x.

Plate 2.9 C, D

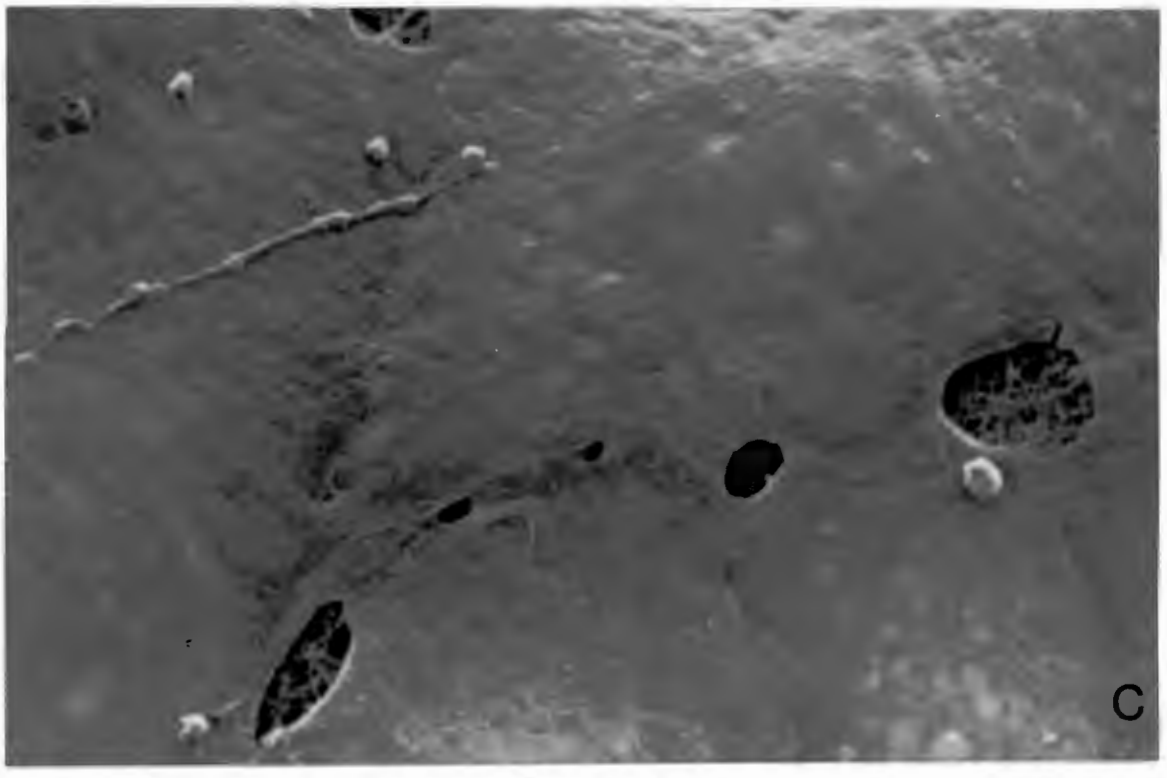


PLATE 2.9 C: Specimen details as for PLATE 2.9 A, B. High power SEM image of E8 intercellular margins revealing apertures through which the underlying fibrillar basement membrane can be seen. Magnification = 8 100x.

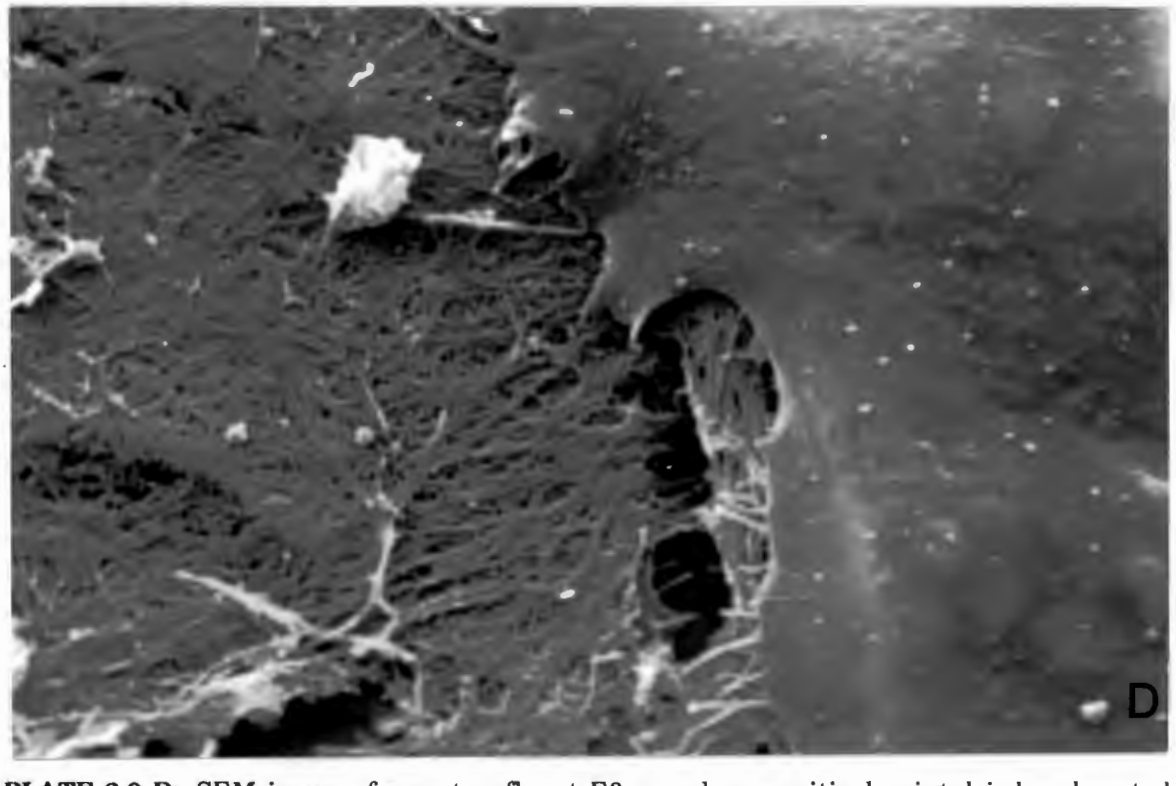


PLATE 2.9 D: SEM image of a postconfluent E3 monolayer: critical point-dried and coated with carbon and gold/palladium. Cells have been lost, thus exposing the underlying fibrillar basement membrane (B). Magnification = 8 100x.

Contact-Inhibition: Autoradiography

Confluent monolayers were quiescent with respect to DNA synthesis by autoradiographic determination of their thymidine indices. E7 and E8 cells (PLATE 2.10 A, B) had indices of 1.62 ±1.52 (S.D.) and 4.87 ±2.30 (S.D.) respectively, expressed here as percentages.

The degree of contact-inhibition of E7 cells, as expressed by thymidine indices, was similar to published values for HUVECs (approximately 3%)[93, 126] while for E8 cells it was slightly higher. It should be noted that, in contact-inhibited cultures, labelled nuclei occurred in small patches and were not uniformly distributed. amongst unlabelled nuclei. This is illustrated in PLATE 2.10 B, a light microscopic autoradiogram of such an active patch in a postconfluent E8 culture. Very large areas existed in which no nuclei were labelled, which was taken to indicate excellent contact-inhibition (PLATE 2.10). Biochemical methods for measuring the degree of contact-inhibition give an integrated result for the entire dish: areas of differing mitotic activity cannot be resolved. Cytochemical methods, however, can discriminate between areas of high and low activity as can be seen by comparing PLATES 2.10 A and B.

Ross[223] discussed the value of cultured ECs as models for the intima within the context of atherogenesis. He suggests that the baseline level of cell division is relatively high in contact-inhibited cultures so that they might behave more like slightly injured than normal intimal ECs. In the present study, the degree of contact-inhibition (as determined by thymidine indexing) has varied widely among different EC cultures. As mentioned above, a single culture may have totally quiescent regions and "hot-spots" in coexistence. It is these silent or quiescent areas that are of interest for modelling the intima because they may more closely reflect the natural state of endothelial cells.

Plate 2.10 A, B

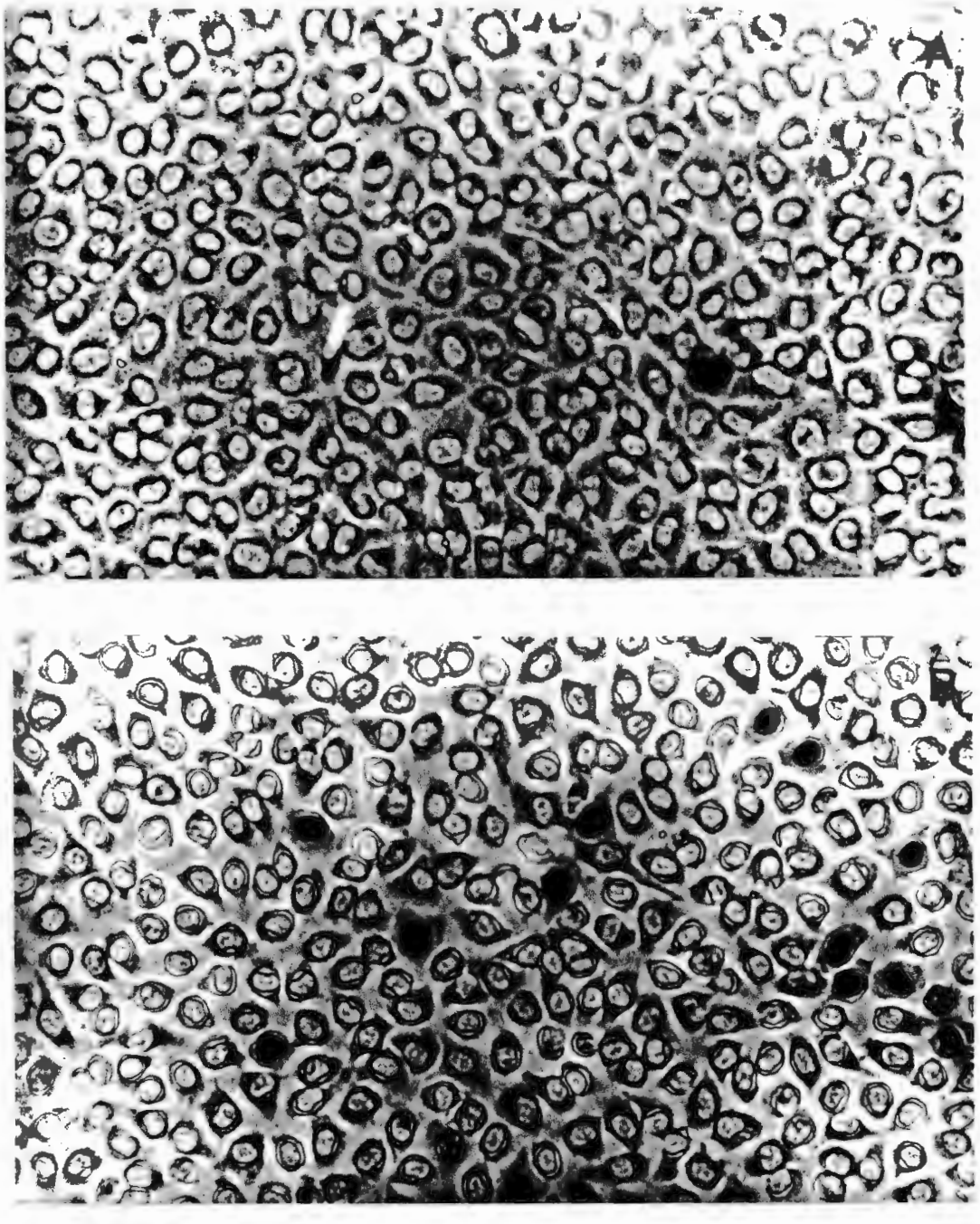


PLATE 2.10: Light microscopic autoradiograms of postconfluent, contact-inhibited cultures of E8. Cells were incubated with |methyl-^{3}H|-thymidine at 2.0μCi/ml in the medium for 18 hours. Ilford K.5 emulsion. Exposure time = 10 days. Phase contrast micrographs. Magnifications A, B = 320x.

A: Quiescent region of monolayer. DNA synthetic activity is extremely low as only a single nucleus has silver grains (indicating radioactive thymidine incorporation) associated with it. The mitotic activity in this field was virtually zero over the 18 hour period of incubation with radioactive thymidine.

B: Isolated small regions of cells active in nuclear DNA synthesis were occasionally detected. Such regions were responsible for the overall mitotic indices of E8 being approximately 5% (see Materials and Methods).

Growth Characteristics

Growth Curves

Figure 2.1 depicts matched growth curves for E3 seeded at 1500 cell/cm^2 on plain and gelatin-treated plastic dishes. Plain plastic produced a monolayer of higher final cell density than gelatin-treated surfaces. Typical logarithmic growth kinetics prevailed 24 -48 hours after plating until maximum density of cells was achieved, around day 14. These cultures were by then postconfluent having reached morphological confluence after about 7 days. E8 cells were generally more vigorous and required only 3-4 days after confluence to reach maximum or saturation density. If cultures were monitored for longer than two weeks, the cell numbers oscillated below saturation density whilst cell loss and regrowth cycling occurred.

Fibroblast Growth Factor

The effect of FGF supplementation on the growth of ECs seeded at low density was examined. E3, when plated at 3500 cells/cm^2 on plastic dishes and supplemented with FGF, grew more rapidly and reached a higher saturation density (1.5x10p5 cells/cm^2) than controls without FGF (1.2x10p5 cell/cm^2) E3 produced contact-inhibited monolayers in the absence of FGF and had a tendency to develop sprouters about 10 days post-confluence (PLATE 2.3). E8 was also FGF-independent.

Conclusion

The bovine aortic cells isolated and used in this study were identified as endothelial cells on the basis of cell and cultural morphology. They grew to become obligate contact-inhibited monolayers which was established by autoradiographic asssessment of DNA synthesis (thymidine index). The monolayers were polarised: the apical aspects which faced the medium expressed microvilli and microprojections while the basal aspects were associated with basement membrane, secreted by the cells. Confluent monolayers expressed vWF (Factor VIII-related) antigenicity, the main identificatory criterion for ECs. In addition they possessed the ultrastructural characteristics of ECs: abundant endothelial vesicles, intercellular junctional complexes and fibrillar extracellular matrix on their basal aspects (basement membrane).

The bovine aortic EC culture system chosen compared favourably with aortic ECs in vivo (reviewed in Chapter 1), having many points of similarity with them. Contact-inhibited monolayers with typical EC morphology and ultrastructure required no specialised substrata and grew successfully on untreated plastic dishes. In addition, the cultures were independent of FGF additions to the medium for the expression of typical contact-inhibited monolayers. A point of dissimilarity between the ECs in culture, as used here, and ECs in the endothelium was the absence of clearly identifiable gap and tight junctions. As mentioned before, this was probably as a result of the repeated passaging of cells, which can result in the progressive loss of expression of these specialisations. Thus, where transport experiments are envisaged, primary[162] EC cultures of endothelium in situ[280, 257] which have these junctions, are indicated. However, the normal expression of tight and gap junctions was not essential to the validity of the EC model as used in this study of LDL receptor expression and distribution.

CHAPTER THREE

LDL and AcLDL Interactions with Active and Quiescent Endothelial Cells: Fluorescence Microscopy and Radiochemical Labelling

INTRODUCTION

LDL

Three mechanisms are known for the endocytosis of LDL in ECs: high-affinity receptor-mediated endocytosis,[210] low-affinity adsorptive endocytosis[279] and fluid phase endocytosis or pinocytosis.[67, 279] The high-affinity route is thought to provide the ECs with cholesterol for synthesis of new membranes[77] while pinocytosis and low-affinity adsorptive endocytosis can transport LDL across the cytoplasm.[280] Pinocytosis, low-affinity routes as well as high-affinity routes may also have other functions like retro-endocytosis[9] and degradation[210, 267] of LDL in ECs. Transport by transendothelial routes, or transcytosis,[280] takes place via the endothelial vesicular pathway. This transport mechanism may involve discrete moving vesicles[57, 58] or transendothelial channels[256] or both.[84, 85, 86] The ultrastructural basis of transcytosis has been discussed in Chapter 1. Data obtained in vivo[29, 269] and in situ using aortic preparations[266, 280] indicated that transcytosed LDL particles remained relatively intact. These LDL may be catabolised by non-ECs underlying the intima.[280, 279, 67, 264, 266]

The rate of LDL endocytosis and degradation via all three routes is coupled to the morphological state of ECs in culture. Confluent cells endocytose and degrade far less LDL than actively growing cells.[286, 60, 240] This includes uptake by pinocytosis and low-affinity adsorptive routes which far outweigh the contribution of high-affinity receptor-mediated routes at physiological concentrations of LDL.[60, 279] Thus receptor-independent routes of uptake of LDL are markedly reduced in postconfluent endothelial cultures although the regulatory mechanisms are unknown.

High-affinity receptor-mediated endocytosis of LDL, considered apart, is also extremely sensitive to the morphological state of cultured E C. Confluent, contact-inhibited monolayers in vitro endocytose up to ten-fold less LDL by high-affinity receptors than do actively-dividing subconfluent cultures.[286, 279, 240] Such monolayers, quiescent with respect to LDL metabolism mediated by high-affinity receptors, may be analogous to the endothelium in vivo.[131] Carew et al.[47] and Wiklund et al.[294] have reported markedly reduced receptor-mediated LDL flux in rabbit aortic intima in vivo. Minimal receptor-mediated endocytosis of LDL was previously reported in postconfluent bovine EC cultures.[284, 286] Those studies, based on the metabolism of ^{125}I-LDL[286] and immunofluorescence microscopy,[284] led to the suggestion that the uptake of LDL was severely limited by morphological constraints inhibiting endocytosis of surface-bound LDL. It was suggested by Vlodavsky et al. that the LDL receptor in dividing ECs is free to diffuse laterally leading to aggregation and endocytosis, whereas in postconfluent ECs lateral diffusion of receptors appeared to be restricted which inhibited internalisation.[283]

Reduced LDL metabolism in postconfluent ECs has also been shown by others using bovine[60] and human[60, 279] E C. But in those studies[60, 279] the effects of cell density on the efficiency of LDL internalization were not reported. Efficiency of internalisation was shown by Strumpfer[271] to be independent of the cell density in bovine aortic ECs in culture. Although binding levels became markedly depressed in cultures approaching confluence, the efficiency of internalisation was unaffected. This trend continued until levels (number of LDL receptors) became so low that high-affinity receptor-mediated uptake measurements became insensitive in postconfluent ECs.

In this chapter, LDL binding and internalization in dividing bovine ECs have been compared with that of postconfluent ECs exhibiting strict contact-inhibition. Light microscopy has been used here to describe the topographical and intracellular distribution of LDL rendered fluorescent either by immunocytochemistry or by direct labelling. This served two purposes: first to extend and confirm previous findings[271] concerning the level of LDL bound by contact-inhibited monolayers, and second, to re-examine the question of restricted lateral mobility of LDL receptors in postconfluent as opposed to actively-dividing E C.[284]

In this study use was made of LDL directly labelled with a lipid-soluble fluorescent dye, 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI) which can be detected by fluorescence microscopy. This had the advantage over indirect immunofluorescence techniques that DiI-labelled LDL could be used to follow all stages of binding and endocytosis without permeabilization of membranes to allow antibody access to intracellular sites. Indirect immunofluorescence was however also used to repeat previous experiments,[284] to validate the behaviour of DiI probes, and to describe surface binding at 37°C where directly-labelled probes would be unsuitable. All fluorescence studies were complemented by radiochemical assays of lipoprotein binding levels, intracellular levels, and degradation rates.

The findings are interpretted here using current concepts of the control of the LDL receptor pathway via the regulation of receptor synthesis.[35, 231, 96] It is argued that the regulation mechanism for LDL uptake in cultured bovine aortic ECs is similar to that of other cell types in which LDL homeostasis has been investigated, e.g. human skin fibroblasts[34] and smooth muscle cells.[20] Markedly reduced receptor numbers on postconfluent cells, which have little demand for cholesterol, appear to account for the proportionately reduced uptake of LDL when compared with actively-growing cultures with high cholesterol demand.

Although the monolayers used in the studies of Vlodavsky et al.[284, 286] were dependent on FGF for contact-inhibition, equally well contact-inhibited monolayers have been successfully grown without FGF[240, 70] (see Chapter 2, Growth Factors). Using FGF-independent postconfluent monolayers as an endpoint, the modulation of high-affinity LDL receptor-mediated endocytosis as the cultures progressed from active division to quiescence is considered in this chapter.

Acetylated LDL

ECs possess a distinct binding site for acetylated LDL (AcLDL)[265] similar to the receptor found on macrophages whose scavenging function for modified-LDL is now well characterized.[33, 102]

Modified-LDL may play an important role in atherogenesis particularly in overloading of macrophages with cholesteryl esters[182] (see Chapter 1). The question of the role of ECs in atherogenesis arises since they too have the receptor for modified-LDL, and can also bring about the modification in vitro.[182] ECs from rabbit and bovine aortae, possess distinct receptors for modified LDL, βVLDL and native LDL,[13] like monocyte-derived macrophages. Macrophages and ECs are involved in the early stages of atherogenesis and it is tempting to implicate their unique capacities for receptor-mediated uptake of both βVLDL and modified-LDL in this process. Lipid-loaded SMC are also found in fibrous atherosclerotic plaque,[75, 74, 224, 225] where they too probably become foam cells. Smooth muscle cells migrating out into a damaged area of the intima[224, 225] may come into direct contact with newly modified LDL from either macrophages or ECs, also present in the lesion area (see Chapter 1, Atherosclerosis).

Strumpfer[271] found that although the AcLDL receptor in cultured bovine E C was not subject to regulation, the cells never accumulated excessive amounts of cholesterol ester under any conditions. This appears to argue against the direct injury of ECs by lipid overload via modified LDL receptor-mediated endocytosis. It also raises questions as to the role of modified-LDL receptors in postconfluent ECs as they exist in vivo.

The surface distribution of AcLDL receptors has only recently been described on macrophages by fluorescence[277] and transmission electron microscopy.[218] Their distribution on postconfluent ECs has been unknown until recently.[240, 128] Traber et al.[276] using indirect immunoperoxidase techniques at the EM level found the AcLDL receptor in human monocyte-derived macrophages to be diffusely distributed on the surface of cells incubated with AcLDL after fixation. In preparations incubated with AcLDL before fixation, ligand-induced clustering of receptors was revealed. In contrast to these results, an investigation by Van der Schroeff et al.[277] of the binding of AcLDL to cultured porcine monocytes at 4°C and 37°C failed to

demonstrate specific AcLDL binding either by immunofluorescence microscopy or electron microscopical immunocytochemistry. They concluded that, because AcLDL could be detected biochemically, the morphological binding pattern was probably diffuse and not amenable to detection by indirect immunocytochemistry. They were able to demonstrate clustered receptors binding LDL under the same experimental conditions. The ultrastructural methods of both the above studies were limited to the analysis of ultrathin sections which reveal only cell profiles. This methodology may not be optimal for topographical analyses (see Chapter 4, pages 120, 159). Robenek[218] made a topographical study of the distribution of AcLDL receptors in mouse peritoneal macrophages by preparing high resolution surface replicas for analysis by transmission electron microscopy. His results corroborated those of Traber et al.[276] by showing a diffuse distribution at 4^{0}C at which temperature the lateral diffusion of receptors is severely limited (analogous to pre-binding fixation). A typical coated-pit clustering occurred when cells were warmed for 8 minutes after ligand binding (analogous to post-binding fixation).

There are similarities between macrophages and ECs in the expression of AcLDL receptors analysed biochemically. But little is known of the morphological nature of AcLDL receptors as expressed in ECs. Consequently their topographical distribution has been visualised by fluorescence microscopy in the study reported here. The distribution of AcLDL on the surface of ECs in the subconfluent and postconfluent morphological states is described. As for LDL, the modulation of high-affinity receptor-mediated binding and endocytosis of AcLDL has been described morphologically as EC cultures progress from active growth to the contact-inhibited, quiescent state. The surface binding and intracellular levels, and the degradation rates were monitored radiochemically using ^{125}I-AcLDL at all stages of the morphological analyses.

Strumpfer[271] and Stein and Stein[265] have shown that the modulation of receptor numbers for AcLDL on ECs is opposite to that for LDL receptors, as the cultures mature from active growth to contact-inhibition. Markedly fewer AcLDL receptors were detected in active, dividing cells than in confluent, contact-inhibited cells. It is clear that receptor-mediated endocytosis and degradation of AcLDL can occur efficiently in postconfluent ECs which take up miniscule amounts of LDL by this route. These findings which were based on biochemical analyses have been extended on a morphological basis by the fluorescence microscopical analysis presented here.

Dividing cells in culture have been used by others as a model for the regeneration of damaged endothelium.[93, 248, 126] The potential for regeneration in vivo after pathologically or experimentally induced loss of E C by wounding, expresses as re-endothelialisation of denuded areas by migration and spreading of cells at the wound edge. If the wound area is too extensive to be covered in this way, then previously quiescent cells will start to divide to fill the space.[248, 93, 221] By analogy, contact-inhibited and actively-dividing cultured bovine ECs have been used to study the interactions of normal and "wounded" (or regenerating) endothelium with LDL and acetylated LDL in vitro. To strengthen the analogy, postconfluent monolayers were artificially wounded to demonstrate the differential responses of quiescent and regenerating cells to LDL and AcLDL in the same culture. This methodology tests the reversibility of the modulation of receptor expression in postconfluent cells.

MATERIALS and METHODS

Cells and Culture Techniques

Foetal bovine aortic ECs lines E3 and E8 were used for the fluorescence and biochemical experiments described. ECs were isolated and characterised as described in Chapter 2. Seeding densities of 7500 cell/cm^2 were used in all experiments to produce actively-dividing subconfluent cultures in 1 to 3 days (see Chapter 2, PLATE 2.2). If allowed to continue growing, ECs reached confluence within 8 days (see Chapter 2, PLATE 2.1 and PLATE 2.9 A). Contact-inhibition was monitored by autoradiography (Chapter 2)

Normal human skin fibroblasts GM 0203 and GM 3348 were used as positive controls for LDL receptor binding studies. GM 0701 and GM 2000, LDL receptor-negative mutants which do not bind LDL, were used as negative controls.

Details of the sources, characteristics and culture methods of all the cells discussed in this chapter were given in Chapter 2, pages 35-37.

Biochemical Procedures

Preparation of Lipoproteins and Lipoprotein-Deficient Serum

Human LDL (d = 1.019-1.063 g/ml) and human lipoprotein-deficient serum (d > 1.25 g/ml) (LPDS) were isolated from the serum of male normolipidaemic donors by ultracentrifugation in a Beckman L8-70 ultracentrifuge (Beckman Instruments Inc., Fulleston, California). Fractions were banded by discontinuous gradient centrifugation.[55] 13 ml aliquots of serum, adjusted to density 1.30 gm/ml with solid KBr, were layered at the bottoms of quick-seal tubes containing 27 ml of saline-EDTA (0.85% NaCl + 0.01% EDTA, pH 7.0). These were sealed and ultracentrifuged in a Beckman VTi50 rotor at 50 000 rpm for 2.5 hours at 10^{o}C. After isolation of the lipoprotein fractions with the aid of a tube-slicer, the LDL fraction was washed at density 1.063 gm/ml by recentrifugation in a Ti60 rotor at 50000 rpm for 16-18 hours at 10^{o}C. LPDS fractions were washed at density 1.25 gm/ml in a VTi50 rotor at 50 000 rpm for 6-8 hours at 10^{o}C. The final bands of lipoproteins were collected using the tube slicer, dialysed against saline-EDTA and sterilised by microfiltration. The concentrations of lipoproteins were expressed in terms of their protein content as determined by the standard method of Lowry et al.[168]

LDL were acetylated using acetic anhydride according to the method of Fraenkel-Conrat.[82] Briefly, equal volumes of freshly prepared LDL and cold, saturated aqueous sodium acetate were mixed and kept on ice for 5 minutes. Acetic anhydride (1 µg/mg LDL protein) was added dropwise, with mixing, over a period of one hour. The resulting acetylated LDL (AcLDL) was dialysed overnight against saline-EDTA, and then centrifuged at 7000 xg in a Beckman Microfuge for 2 minutes to remove precipitated material. The standard method of Lowry et al.[168] was used to determine the protein content of AcLDL. Yields of approximately 90% of the initial protein were obtained after acetylation of LDL . The concentration of AcLDL was always expressed in terms of its protein content. AcLDL was sterilised by microfiltration, stored at 4^{o}C and used within 3-4 weeks. It was necessary to centrifuge the AcLDL to remove precipitates before experimental use.

LDL and AcLDL were iodinated with $Na^{125}I$ (Radiochemical Centre, Amersham, England) using the iodine monochloride method as modified by Bilheimer et al.[22] The specific activities obtained were 200-400 cpm/ng protein.

Binding, Uptake and Degradation of ^{125}I-LDL and ^{125}I-AcLDL

Biochemical procedures were carried out on cultures in styrene tissue culture dishes (Sterilin, Teddington, Middlesex, England). Where indicated the lipoprotein receptor activity of ECs was upregulated[20, 32] by exposure of the cells to MEM-LPDS (MEM containing 5 mg heat-inactivated human LPDS protein /ml) for 48 hours. The cell layers were briefly rinsed with MgPBS to remove traces of growth medium (MEM-FCS) before MEM-LPDS was added. After 24 hours the MEM-LPDS was renewed.

The surface-binding of LDL and AcLDL to cells at 4°C was assayed by incubating them in Medium A (MEM buffered with 10 mM HEPES, pH 7.4, containing 5 mg LPDS protein/ml) containing 20µg /ml of the appropriate radio-iodinated lipoprotein in the presence or absence of excess (200µg /ml) of the homologous unlabelled lipoprotein for 2 hours. Since both LDL and AcLDL binding involve high-affinity receptors and low-affinity sites, the diluting effect of the excess unlabelled ligand allowed accurate determination of the receptor-mediated process. To obtain the high-affinity receptor bound fraction, the low-affinity fraction (amount of radiolabelled ligand bound in the presence of excess unlabelled ligand) was subtracted from the total ligand bound by both processes (amount of radiolabelled ligand bound in the absence of unlabelled ligand).

A ligand concentration of 20µg /ml strongly favours receptor-mediated binding of LDL and AcLDL in ECs; and of LDL in human skin fibroblasts. Strumpfer[271] gave the Kd for LDL on bovine aortic ECs (E3) as approximately 20µg protein/ml, and for AcLDL as approximately 2µg/ml, regardless of temperature. For normal human skin fibroblasts, the Kd for LDL is less than 2µg protein/ml at 4°C and more than twice this at 37°C.[99] So under the present experimental conditions, ligand concentrations were below (LDL) or slightly above saturation (AcLDL) levels, but within the range where receptor-dependent binding, endocytosis and degradation predominated. Typically, at LDL concentrations in the range 20 - 50µg protein/ml of LDL, subconfluent E8 cells endocytosed and degraded 80-90% of the ligand by receptor-mediated processes.

After ligand binding the cells were washed at 4°C, 4 times with MgPBS containing 0.2% w/v BSA (Fraction V, Sigma Chemicals, St Louis, USA) (PBS-alb), and thrice with MgPBS to remove non-specifically bound lipoproteins.[99, 271]

Since lipoproteins bound by incubation with cells at 4°C are not internalized or degraded, the radioactive material associated with the cells was taken as a measure of surface-binding.[99] Cells were dissolved in 1M NaOH; assayed for ^{125}I radioactivity and the protein determined.[168]

Apart from surface-binding, the analysis of lipoprotein metabolism in ECs at 37°C included the assay of intracellular levels and degradation rates. After 4 hours incubation at 37°C in MEM-LPDS containing lipoproteins (concentrations as indicated above for 4°C), cells were cooled on ice for 10 minutes and the medium collected. After washing to remove non-specifically-bound material (as above), surface-bound lipoproteins were assayed as ^{125}I-radioactivity releasable from intact monolayers. Neither dextran sulphate nor heparin[99] released AcLDL efficiently from the surface of bovine aortic ECs (line E3).[271] Surface-bound ^{125}I-AcLDL and ^{125}I-LDL were assayed as radioactivity released from intact monolayers by trypsinisation. Dishes were incubated with 1 ml of 0.05% trypsin containing 0.02% EDTA for 20 minutes at 4°C, during which time time the cells detached from the dishes. The trypsin proteolytic reaction was arrested by the addition of 1ml of MEM-LPDS to prevent damage to the cells. The suspended cells were centrifuged at 1000 xg for 10 minutes and the supernatant fluid (binding) assayed for radioactivity. A disproportionately high level of ^{125}I-LDL saturable binding was repeatedly observed in sparse cultures. This might have reflected binding of ligand to the exposed areas of the plastic culture dish. After removal of the supernatants, ^{125}I-LDL and ^{125}I-AcLDL-treated, pelleted cells were washed twice by recentrifugation in MgPBS and assayed for intracellular radiolabelled lipoproteins. Cell

pellets were extracted with 40% chloroform solution in methanol and centrifuged at 1000xg for 10 minutes. The pellets were dissolved in 1M NaOH at 37°C overnight. From aliquots of this solution radioactivity was counted and protein determined.[168] The amount of degraded material present in the medium was assayed according to the methods of Bierman et al.[21] Briefly, TCA-soluble radioactivity, representing degradation products of the lipoproteins, was counted after free iodine extraction. All ^{125}I-labelled samples were counted in a Packard PGP Autogamma (PRIAS) counter.

Fluorimetric Determination of Uptake of DiI-labelled LDL

The fluorescent dye 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI) was incorporated into LDL and AcLDL as described (page 73) to produce DiI-labelled lipoproteins.[14] Like radio-iodinated lipoproteins, these fluorescent lipoprotein analogues could be used as biochemical probes for the study of lipoprotein metabolism. Cells were incubated with DiI-LDL (20µg/ml) at 37°C for 4 hours. Where necessary, excess unlabelled LDL was added to the dishes at a concentration of 300µg/ml. After standard washing at 4°C as described above, cells were scraped from 50 mm diameter culture dishes with a rubber policeman and washed twice in PBS with centrifugation at 4°C. The pellets were extracted overnight with 1 ml of methanol. After centrifugation, the DiI in the supernatants was determined fluorimetrically as described under Direct Fluorescence Methods (p.74)

Fluorescence Microscopy

Fluorescence experiments were conducted initially on cells cultured on glass slides (Lab-Tek Tissue Culture Chamber/Slides, Miles Laboratories Inc., Naperville, Illinois, U S A). The immunocytochemical procedures used for labelling intracellular or extracellular antigens in cultured cells involved repeated exchanges of liquids over protracted periods of time. Fibroblasts and ECs detached and were lost progressively from the glass surface throughout immunocytochemical processing. The unavoidable turbulence accompanying fluid exchanges also contributed to cell loss. Cells adhered more firmly to plastic than to glass substrata, as shown (in comparative immunocytochemical experiments) by the greater numbers of cells still attached at the end of the procedures. So most of the experiments reported here were conducted on cells growing on plastic substrata.

Cells were cultured in MEM (supplemented as described in Chapter 2, p.36, but without FGF) for all fluoresence experiments. Where necessary, lipoprotein receptors were upregulated with MEM-LPDS as described under Binding, Uptake and Degradation of ^{125}I-LDL and ^{125}I-AcLDL, p.69

A low temperature pre-incubation of unfixed cells was designed to arrest LDL and AcLDL receptors in whatever pattern they expressed at the time of cooling. Subsequent binding of ligands to these cooled unfixed cells was unlikely to disturb the pattern of receptors because of the reduced membrane fluidity at low temperature (see DISCUSSION, pp. 113-114).

Indirect Immunofluorescence Procedures

Cells were incubated with human LDL at 4°C or 37°C. The LDL was mixed with MEM-LPDS for 37°C incubations or with Medium A (see p.69) for 4°C incubations. The possibility exists that large membrane proteins, like receptors, might be cross-linked and redistributed by divalent antibodies bound either directly or via their ligands. This artefactual patching may be prevented by fixation of the membrane preparations after ligand binding but before immunocytochemistry.

Once LDL had been bound to the surface of cells, did the position of the fixation step in the protocol, before or after the immunofluorescence procedure, have any effect on the fluorescence patterns of bound LDL? This methodological question was answered by comparing the methods of Vlodavsky et al.[284] , who fixed after immunocytochemistry, and Anderson et al.[8] , who fixed just after ligand binding. Human skin fibroblasts GM 0203, expressing normal LDL receptors, and GM 0701, having no receptors were used in the comparison. The method of Anderson et al.[8] was applied as given below. The cells were labelled with LDL (100µg/ml) at 4°C and 37°C. If binding was at 37°C, cells were chilled at 4°C for 10 minutes. The cells were washed 4 times with MgPBS-alb and 3 times with MgPBS at 4°C to remove non-specifically-bound lipoproteins. Cells were fixed with 3% paraformaldehyde with MgPBS for 10 minutes at 4°C and a further 10 minutes at 20-25°C. Cultures were washed thrice (5 minutes each time) in MgPBS before incubation for 45 minutes at 37°C with rabbit anti-human apoprotein B immunoglobulin (RAAB) (Behringwerke, Marburg, FRG) diluted 1:5 in MgPBS. This was followed by four 15 minute washes with MgPBS and incubation for 45 minutes at 37°C with fluorescein isothiocyanate (FITC) conjugated to anti-rabbit immunoglobulin (IgG) antibodies raised in goats (FITC-GAR) (Behringwerke) diluted 1:10 with MgPBS. A method based on that of Vlodavsky et al.[284] was applied which differed from the method of Anderson et al.[8] in the following ways. Fixation followed the incubation with secondary (fluorescent) antibody, and up to this point the procedure was conducted at 4°C. Fluorescence microscopy revealed no essential differences in the surface distribution of LDL on the GM 0203 and GM 2408 processed by the two protocols while GM 0701 was not specifically labelled by either. A detailed description of the binding patterns of LDL to normal fibroblasts is given in RESULTS. Therefore, for the convienience of working at room temperature, the method of Anderson et al.[8] was adopted for all indirect immunofluorescence procedures used for detecting LDL or AcLDL .

The problem of non-specific binding of fluorescent antibodies in immunofluorescence microscopical localization of LDL is well known.[8, 14] A diffuse fluorescent background unrelated to specific antigens is produced which may seriously interfere with the specific fluorescence emanating from the antigenic sites. To solve this problem, in some experiments, fluorescent secondary antibody (FITC-GAR) was preadsorbed against cells identical with those to be used for incubations with lipoproteins. Seven times the area of cells to be used in the experimental incubations was used in these attempts to adsorb out extraneous antibodies which might bind non-specifically to any antigens other than the primary antibody (RAAB). The cells were washed free of medium with MgPBS and the diluted FITC-GAR was incubated with them for 1 hour at 4°C. The pre-adsorbed FITC-GAR was centrifuged to remove cellular debris and used for immunocytochemistry. This practice, while definitely reducing the background, also reduced the specific fluorescence intensity. Fluorescent antibodies specific for the primary antibody probably also bound non-specifically to cell surfaces and so became depleted. Since indirect immunofluorescence was used mainly to validate the use of directly fluorescent lipoproteins (see below), and because the background fluorescence never interfered with the interpretation of specific fluorescence, RAAB was used without preadsorption.

Negative controls were incorporated into the experimental design by omitting various components in the immunocytochemical regime, whilst keeping the rest unchanged. To control for non-specific binding of GAR-FITC to the lipoprotein ligand, the primary or ligand-specific antibody (RAAB) was omitted. To control for non-specific binding of RAAB to the cell surfaces the ligand was omitted. And to control non-specific binding of GAR-FITC to the cell surfaces, both LDL and RAAB were omitted. Finally to control for specific binding of lipoprotein to its

receptor, the entire immunocytochemical procedure, including lipoprotein incubation, was applied to cells incapable of binding LDL, mutant human skin fibroblasts GM 2000 or GM 701, which lack functional surface LDL receptors. Positive controls were human skin fibroblasts GM 0203 and GM 3348 which expressed functional LDL receptors. In all cases, after immunocytochemical processing, cells were thoroughly washed, mounted and viewed with FITC epifluorescence optics exactly as described previously (Chapter 2, p.43).

Photomicrography

Recording FITC fluorescence from labelled surface-bound LDL by conventional photography was difficult. The fluorescence intensity was often very low, and given the rapid photobleaching of the fluorophore, micrography was often only possible using exposures of 20 seconds or more on film rated at 1600 ISO (Ilford XPI). The micrography of AcLDL bound to the surfaces of postconfluent ECs and visualised by indirect immunofluorescence was particularly difficult. To do justice to it, an image intensification system would have been required. Such a system was unavailable in South Africa.

Triton Permeabilisation of Cells

In some experiments, LDL or AcLDL was incubated with cells at 37^{0}C in order to study endocytosis. To visualise intracellular LDL and other apo-B- containing molecules, fixed cells were exposed at room temperature to 0.05% Triton X-100 detergent in MgPBS for 10 minutes.[8] This permeabilisation treatment, which partially solubilised the plasma membranes, allowed specific antibodies access to apo-B-containing molecules at intracellular sites. After washing out the detergent (three soaks in MgPBS, 5 minutes each time), LDL-treated cells were incubated with antibodies as described above.

Direct Fluorescence Methods

The fluorescent dye 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate * (FIGURE 3.1) (DiI) (Molecular Probes, Plano, Texas) was incorporated into human lipoproteins to produce the fluorescent lipoprotein analogues or probes.[14, 204]

At least two methods of labelling lipoproteins with DiI have been reported in the literature: that of Barak and Webb[14] and that of Pitas et al.[204] Both methods were attempted. Barak and Webb adapted the methods of Krieger et al.[159] who reconstituted LDL with other fluorescent probes. The methods involved lyophilisation of the lipoproteins in the presence of potato starch, lipid extraction in heptane at -20°C, reconstitution with DiI dissolved in heptane, drying and finally rehydration and ultracentrifugal isolation of the probe. Lipoproteins labelled by this method were examined by transmission electron microscopy after negative staining (Chapter 2. p.40) and found to differ morphologically from their native forms. The particles of DiI-LDL (PLATE 3.1 B) and DiI-AcLDL (PLATE 3.1 D) prepared by the reconstitution method appeared irregular with many discoidal forms, and were often aggregated. In contrast DiI-LDL (PLATE 3.1 A) or DiI-AcLDL (PLATE 3.1 C) prepared by the simpler Pitas method were indistinguishable from native LDL (PLATE 3.2 A, B) and AcLDL (PLATE 3.2 C, D).

Accordingly, DiI-labelled lipoproteins were prepared according to the method of Pitas.[204] Briefly, 1 mg of LDL or AcLDL (on the basis of protein) in 2ml of LPDS was vortexed for 10 -15 seconds with 50µl of a 3 mg/ml DiI solution in dimethyl sulphoxide. The incorporation of DiI into the lipid phase of the lipoproteins proceeded over 8-15 hours at 37°C. Solid KBr was added to the incubation mixture to raise the density to 1.063 g/ml and DiI-labelled-lipoproteins were isolated by ultracentrifugation for 24 hours at 40 000 rpm in a Beckman SW40 Ti rotor. The LDL fraction floating in the top 4-6mm was collected and dialysed against saline EDTA (0.9% NaCl, 0.01% EDTA) for two days. DiI-LDL was sterilised by membrane filtration (0.22µm

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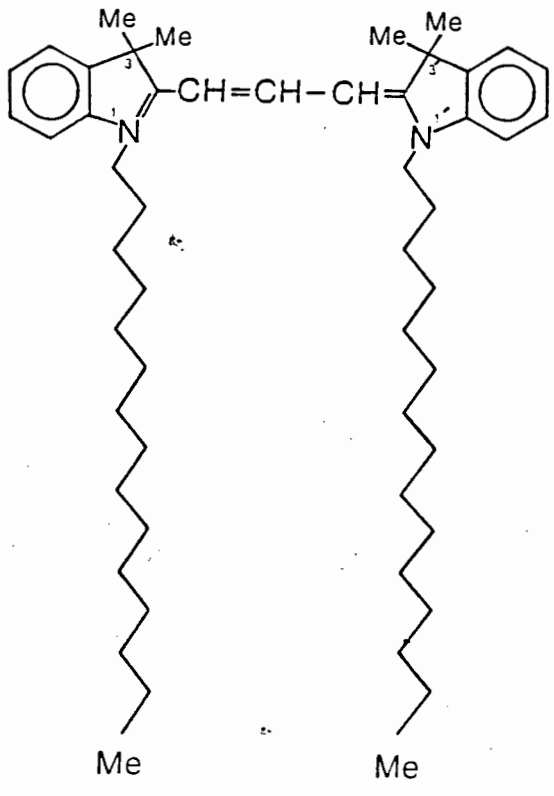
* FIGURE 3.1: THE MOLECULAR STRUCTURE OF DiI



The molecular structure of DiI (Fig.3.1) comprises a pair of indole rings with dimethyl groups at the 3 and 3' positions. These heterocyclic rings are linked via their 2 and 2' positions by a chain of three methine groups. Octadecyl chains are linked to each nitrogen molecule at positions 1 and 1' of the pentacyclic rings. The DiI used here is properly abbreviated DiI(3)C18 : where "Di" refers to the two heterocyclic rings, "I" to indole rings, "3" to the three methine groups and "C18" to the alkyl chains. DiI is a carbocyanine dye on account of the three methine groups. The heterocyclic rings linked by the odd number of methine groups provides the resonance necessary for fluorescence behaviour.[55, 127] The molecule is cationic bearing a single charge on one of the nitrogens. The DiI used in the present study had a perchlorate counterion.

The C18 alkyl chains confer hydrophobicity making the dye soluble in some lipid-rich regions of lipoproteins. Whether DiI dissolves into the lipid core or the phospholipid shell of LDL is unknown. Barak and Webb[14] suggest that since the fluorophore DiI is cationic it will not readily dissolve into the non-polar core of the particle. The long chain saturated fatty acids are also unlikely to incorporate into the core. Spectroscopic comparisons of DiI-LDL with DiI dissolved in various solvents are consistent with a phospholipid association.[14]

Millex GV, Millipore Corporation) before storage at 4°C. The protein concentration of the DiI-LDL was determined by a the method of Lowry et al.[168] using lyophilised aliquots from which the DiI had been extracted in methanol. This procedure avoided the interference of DiI colour with the spectrophotometric determination of protein. The DiI content of the DiI-labelled lipoproteins was determined fluorimetrically from methanol extracts using a Perkin Elmer model 203 spectrofluorimeter (excitation and emission wavelengths, 520 and 570nm respectively). Standard solutions of DiI in methanol were used to calibrate the system.[204] Specific activities of DiI-LDL ranged from 29-59 ng DiI/µgLDL protein and of DiI-AcLDL from 32-57 ng DiI/µgAcLDL protein. SDS solubilised DiI-LDL was run on SDS-PAGE and the Apo B found to comigrate with native Apo-B from SDS solubilised LDL.

DiI-labelled lipoproteins were incubated with cells at 4°C or 37°C. Washing and fixation steps were the same as for immunofluorescence experiments (see above). Where necessary, DiI-LDL was released from cell surfaces by treatment with dextran sulphate (10 mg/ml) for 60 minutes at 4°C. Intracellular DiI fluoresced very brightly and was easily detected through the cellular mass. Preparations were viewed by epifluorescence microscopy (excitation wavelength range 515-560 nm). The emission wavength (580 nm) yielded brilliant orange-red fluorescence. The DiI optics were incorporated into a Leitz Laborlux 12 fluorescence microscope as a dichroic beam splitter module (Ploemopak) comprising a BP 515-560 nm exciter filter, a splitter mirror passing 580 nm to the ocular and an LP 580 barrier filter.

It was possible to observe the green fluorescence from FITC by fluorescence microscopy on DiI/FITC double labelled preparations using FITC optics. In agreement with Barak and Webb,[14] It was found that although the fluorescence emissions of DiI and FITC were easily distinguishable (red-orange and green respectively), residual DiI fluorescence under FITC excitation conditions (450-490 nm) was seen. To minimize this, a green filter (550nm LP) was positioned between the dichroic beam splitter and the ocular in order to suppress any red-orange DiI fluorescence.

Wounding Experiments

Confluent E8 cultures in MEM-LPDS were wounded by making a single streak down the middle of the culture dish with a rubber policeman. This denuded a narrow path of cells without damage to the plastic substratum. Cultures were then incubated for 16-24 hours in MEM-FCS to allow partial regeneration of the damaged endothelial monolayer by migration of cells on the wound edge into the "wound" space. This regeneration was vigorous in cultures incubated with complete medium (MEM-FCS) but slower if MEM-LPDS was used. Lipoprotein interactions with regenerating cultures were studied by direct fluorescence microscopy.

Plate 3.1 A-D

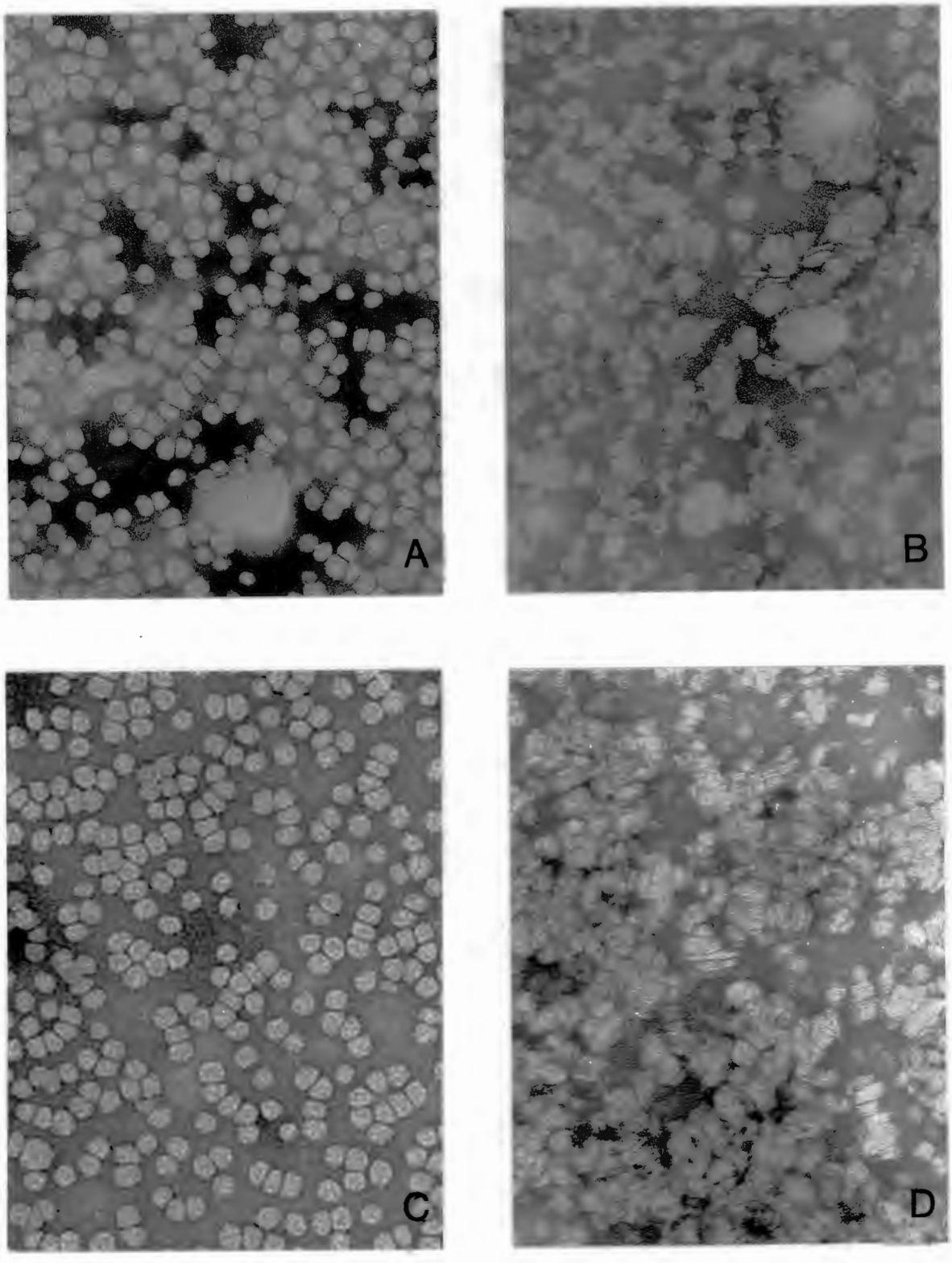


PLATE 3.1: DiI-labelled native and acetylated LDL. Negatively-stained with phosphotungstic acid. Transmission electron micrographs. **A:** DiI-LDL prepared according to the method of Pitas et al.[204] Magnification = 157 000x. **B:** DiI-LDL prepared according to the method of Barak and Webb[14] Magnification = 157 000x. **C:** DiI-AcLDL prepared according to the method of Pitas et al.[204] Magnification = 250 000x. **D:** DiI-AcLDL prepared according to the method of Barak and Webb[14] Magnification = 157 000x.

Plate 3.2 A-D

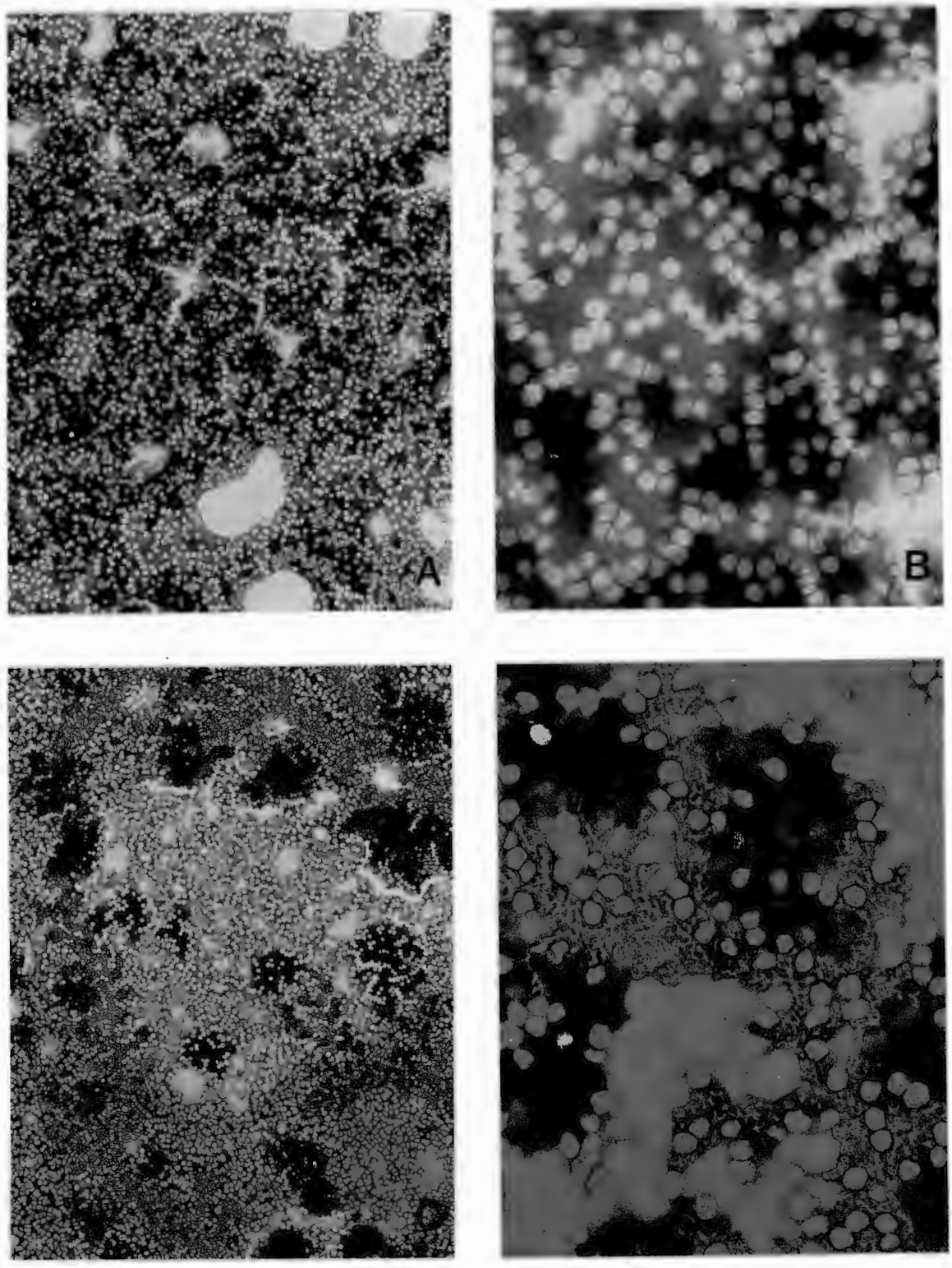


PLATE 3.2: Native and acetylated LDL, negatively-stained with phosphotungstic acid. Transmission electron micrographs. **A, B:** LDL. Magnifications A = 51 000x, B = 180 000x. **C, D:** AcLDL acetylated according to the method of Fraenkel-Conrat.[82] Magnifications C = 36 000x, D = 168 000x. The finer material interspersed with the AcLDL particles is a crystalline substance, probably NaCl from the diluent.

RESULTS

Lipoprotein Receptors

Binding sites for LDL and AcLDL, which satisfy the criteria for receptors, exist on the foetal bovine aortic endothelial cells which were used in the experimental work for this thesis. These binding sites were saturable, of relatively high-affinity and were distinct from one another as demonstrated by competition studies. The two binding sites were independently regulated[271, 240] (see DISCUSSION, p.112).

TABLE 3.1

Exp. No.	Ligand	^{125}I-lipoprotein metabolised (ng/mg cell protein)					
		Bound		Intracellular		Rate Degraded ($4\ h^{-1}$)	
		Sparse	Con-fluent	Sparse	Con-fluent	Sparse	Con-fluent
1	LDL	212	<30a	585	46	1892	30
2	LDL	121	ND	279	ND	1776	ND
3	LDL	296	<20a	346	112	2596	508
	AcLDL	<20a	102	55	426	294	2900
4	LDL	145	28	884	192	2375	766
	AcLDL	ND	ND	28	330	100	1589
5	LDL	155	14	-	-	-	-

a These values represent the minimal measurable limit under the conditions of variation of each experiment

ND Not determined

TABLE 3.1: METABOLISM OF ^{125}I-LIPOPROTEINS BY ENDOTHELIAL CELLS

Sparse and confluent ECs (E8) were prepared by growing cells for 1 day and 6 days respectively as described in MATERIALS AND METHODS, except in the case of Experiment 4, where sparse and confluent ECs were prepared by seeding cells at 2000 cell/cm^2 and 13000 cell/cm^2 respectively, followed by growth for 4 days. Cultures were then treated for 48 hours with MEM-LPDS (renewed after 24 hours) and incubated at 37^{0}C for 4 hours in MEM-LPDS containing 20µg protein/ ml of either ^{125}I-LDL or ^{125}I-AcLDL in the absence or presence of 200µgprotein/ml of the corresponding unlabelled lipoprotein. High-affinity binding, intracellular levels and degradation rates were determined (see MATERIALS and METHODS, p.69). In Experiment 5, LDL binding was determined at 4^{0}C (see MATERIALS and METHODS, p.69).

Effect of EC Morphology on Binding and Metabolism of 125 I- Lipoproteins

LDL

Table 3.1 lists the binding and intracellular levels, and degradation rates of ^{125}I-LDL in subconfluent and postconfluent E8 cultures. The values tabulated represent the extreme values from time-course experiments one of which is detailed graphically in Figure 3.2. The binding levels of ^{125}I-LDL declined as E8 cultures modulated from subconfluent (212 and 296 ng LDL protein/mg cell protein in Expts 1 and 3 respectively) to postconfluent morphology (<30 and <20 ng LDL protein/mg cell protein in Expts 1 and 3 respectively). In agreement with the low binding of ^{125}I-LDL by postconfluent cells (in some cases too low to measure accurately), the intracellular levels and degradation rates of ^{125}I-LDL also decreased dramatically as the cultures reached postconfluent density.

In Experiment 1, postconfluent ECs contained <10% of the intracellular LDL than subconfluent ECs (46 and 585 ng LDL protein/mg cell protein respectively), and degraded only 1.5% of the LDL (30 and 1893 ng LDL protein/mg cell protein) over 4 hours. Although not as impressively, similar trends showed (Experiment 3) in postconfluent ECs. Intracellular LDL was reduced to 32%, and the LDL degradation rate to 20% of those of subconfluent ECs. In all cases, depressed internalisation-dependent parameters (intracellular levels and degradation rates) were associated with low binding values. These trends were progressive with the modulation of cultures from subconfluent to postconfluent morphology (Fig.3.2). With respect to LDL, all receptor-mediated processes (binding, endocytosis and degradation) were minimal by day 6 when contact-inhibition was complete.

AcLDL

AcLDL interactions with subconfluent and postconfluent E8 cultures are also listed in Table 3.1 and shown in Fig. 3.2. The binding levels, intracellular levels, and degradation rates of AcLDL in postconfluent cultures were much higher than in subconfluent cultures. More than five times as much AcLDL was bound by postconfluent ECs than subconfluent ECs (102 and <20 ng AcLDL protein/mg cell protein respectively for Experiment 3)

Similar trends were displayed in the intracellular levels and degradation rates. In Experiment 3, postconfluent ECs contained intracellularly almost 8 times as much AcLDL as subconfluent ECs (426 and 55 ng AcLDL protein/mg cell protein respectively), and degraded almost 10 times as much (2900 and 294 ng AcLDL protein/mg cell protein respectively) over 4 hours. In Experiment 4, very similar trends are shown, although not as clearly as in Experiment 1.

The modulation of binding levels, intracellular levels, and degradation rates for AcLDL as subconfluent ECs grow to confluence. are also shown in Fig. 3.2. The trends were the reverse of those seen for LDL. All AcLDL parameters started out low in subconfluent cultures and increased with the modulation of cells to postconfluent monolayers. Maxima were reached by day 6 when contact-inhibited monolayers were fully elaborated.

These opposing trends in LDL and AcLDL metabolism as cultures matured from the proliferative state to postconfluent quiescence (Fig. 3.2) were associated with morphological state and not time in culture. The same trends were seen when seeding density, rather than time, was varied to yield subconfluent or postconfluent cell cultures of the same age (Table 3.1, Experiment 4). Although cells had spent the same time in culture, LDL metabolism was maximal in subconfluent and minimal in postconfluent cultures. Conversely, AcLDL metabolism was maximal in postconfluent and minimal in subconfluent cultures.

The efficiency of internalisation of ligands by receptor-mediated endocytosis may be described conveniently by an internalisation index. This has been defined as the sum of the intracellular level and the amount degraded, divided by the amount bound.[59] Using the values upon which Fig 3.2 is based, with the exception of LDL metabolism in very subconfluent cultures (day 1), internalisation indices calculated for LDL and AcLDL were similar (approximately 30/4 h on day 3 and day 5).

Contact-Inhibition of Postconfluent Cultures

By the autoradiographic methods (see Chapter 2, p.44), the degree of contact-inhibition of postconfluent EC cultures was expressed as a thymidine index. Postconfluent E7 and E8 cells had indices of 1.62% +- 1.52 (SD)and 4.87% +-2.30 (SD) respectively.

Figure 3.2

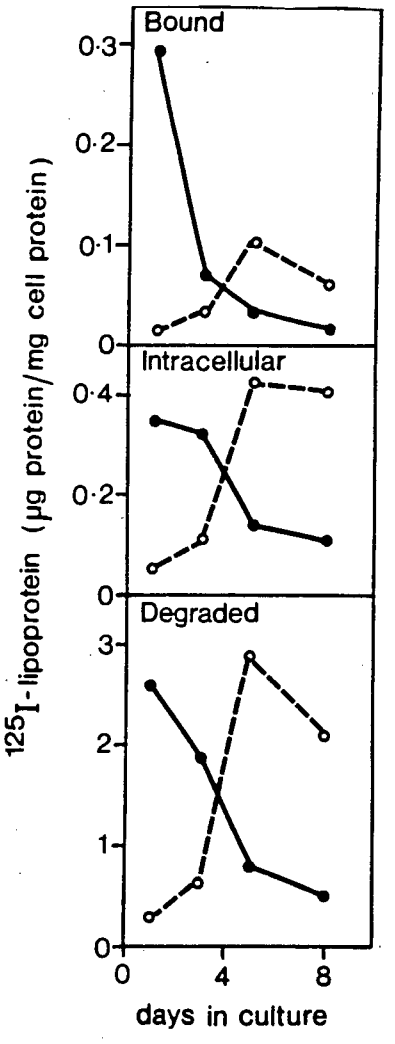


FIGURE 3.2: Metabolism of ^{125}I-LDL and ^{125}I-AcLDL in ECs as a function of time in culture. Endothelial cells (E8) were cultured for the indicated times (see MATERIALS and METHODS). Each dish then received 2 ml MEM-LPDS and was incubated at 37°C for a further 48 h (MEM-LPDS was renewed after 24 h). This medium was then replaced with MEM-LPDS containing 20µg protein/ml of either ^{125}I-LDL (solid line) or ^{125}I-AcLDL (broken line) in the absence or presence of 200µgprotein/ml of the corresponding unlabelled lipoprotein. High-affinity binding levels; intracellular levels; and degradation rates were calculated (see Materials and Methods). Each point represents the mean value of duplicate assays, at each lipoprotein concentration.

Fluorescence Microscopy

In order to standardise indirect immunofluorescence techniques for visualising LDL interactions with cultured cells, human skin fibroblasts were used. Standard receptor assays have previously been applied to human skin fibroblasts.[8, 14] The availability of fibroblast mutants with defective LDL receptors[reviewed 101] allowed additional control systems. Anderson et al.[8] characterised LDL-fibroblast interactions by immunofluorescence microscopy and the results obtained below (with his techniques) agree with his findings.

Normal fibroblasts (GM 0203) bound LDL at 4°C (PLATE 3.3 A,B) and 37°C (PLATE 3.3 C,D) in small punctate foci distributed over the entire cell surface. Sometimes the foci appeared to be arranged in linear arrays shown in PLATE 3.3 D. When LDL was omitted, a diffuse, non-specific background but no focal fluorescence was seen (PLATE 3.12) When mutant fibroblasts (GM0701) which lacked functional DL receptors were incubated with LDL at 4°C, no focal fluorescence was seen.

If normal fibroblasts were incubated with LDL at 37°C, permeabilised by mild detergent treatment and then prepared for immunofluorescence microscopy, large fluorescent foci were seen (PLATE 3.4 A,B). If LDL or the detergent treatment was omitted, the foci were not seen. They were therefore associated with intracellular antigens of LDL. Tritonised cells gave a particularly high non-specific background fluorescence presumably owing to cross-reaction of the primary and secondary antibodies with intracellular antigens exposed by the detergent action.

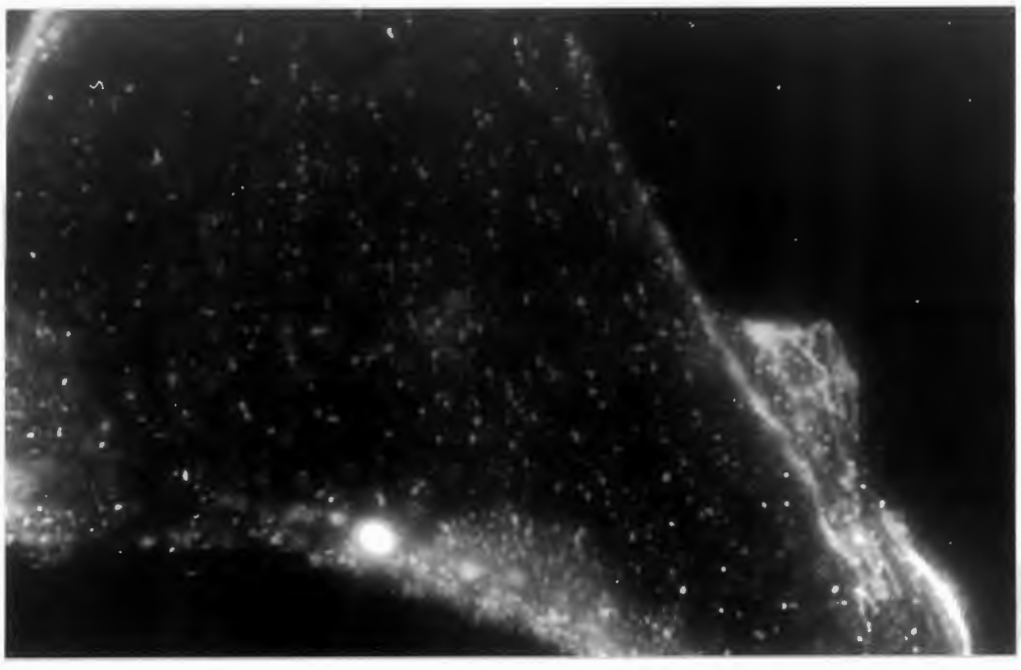
Direct Fluorescence Microscopy

Direct fluorescence microscopy of DiI-labelled lipoproteins gave unequivocal binding data which was uncomplicated by the non-specific binding problems which beset immunofluorescence techniques. The reliability of the DiI-LDL was checked by demonstrating the punctate focal distribution of LDL bound to the surface of human skin fibroblasts at 4°C. On GM 0203 cells this was similar (PLATE 3.5 A) to that seen by indirect immunofluorescence (PLATE 3.5 B). These results confirm those described previously by direct fluorescence (DiI-LDL)[14] and immunofluorescence[8] (LDL) techniques respectively. Receptor-negative fibroblasts, GM 2000 and GM 701, failed to bind DiI-LDL yielding almost totally dark fields.

Intracellular uptake of DiI-LDL at 37°C was seen in GM 3348 as a massive perinuclear accumulation with fluorescence becoming less intense towards the cell periphery (PLATE 3.6 A,B). The fluorescence, like that seen by immunofluorescence techniques, was in the form of large foci (cf. PLATE 3.6 C). These foci are seen more clearly in PLATE 3.6 A.

As an added control procedure, DiI-LDL itself was detected by FITC immunofluorescence. The LDL in the DiI-labelled probe retained Apo B antigenicity against which the primary antiserum, RAAB, was directed. So the ligand was labelled with two different fluorophores each fluorescing at a different wavelength. Each fluorophore was detected individually by means of appropriate filters (see p.74). The DiI and FITC labels co-localized as punctate foci on the surfaces of human skin fibroblasts incubated with DiI-LDL at 4°C (data not shown).

Plate 3.3 A, B



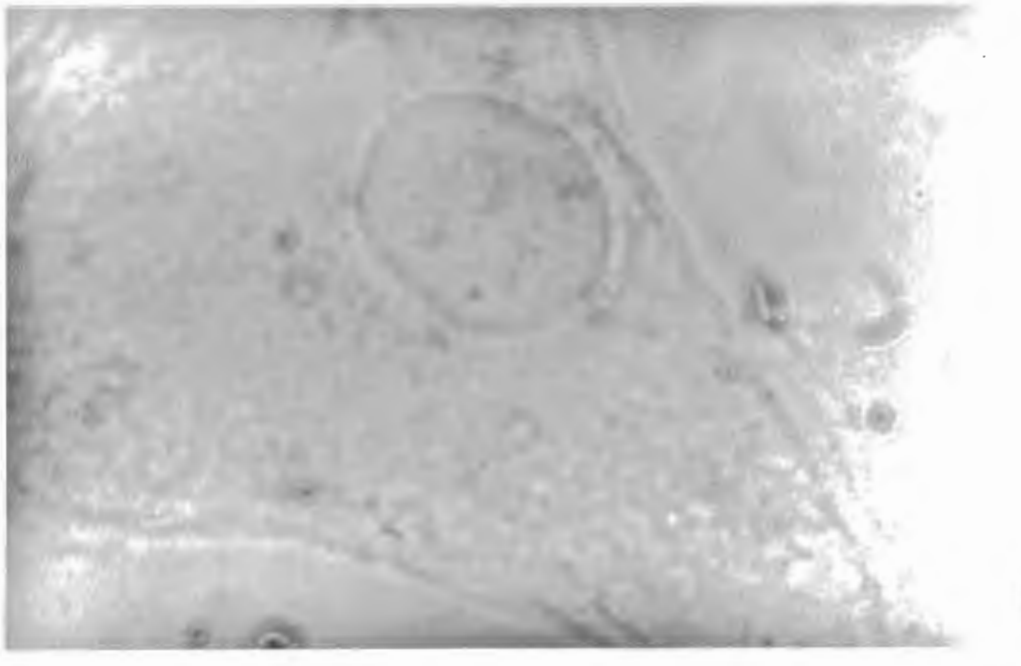


PLATE 3.3: LDL bound to the surface of GM 0203 normal human skin fibroblast cells at 4°C. **A**: LDL receptors have been probed by indirect immunocytochemistry (using LDL, RAAB, and FITC-GAR) and then detected by immunofluorescence in the light microscope. Note the fine, punctate, focal distribution of LDL-specific fluorescence. **B**: Phase contrast micrograph of the same field as shown in A. Magnifications A, B = 1 640x.

Plate 3.3 C, D

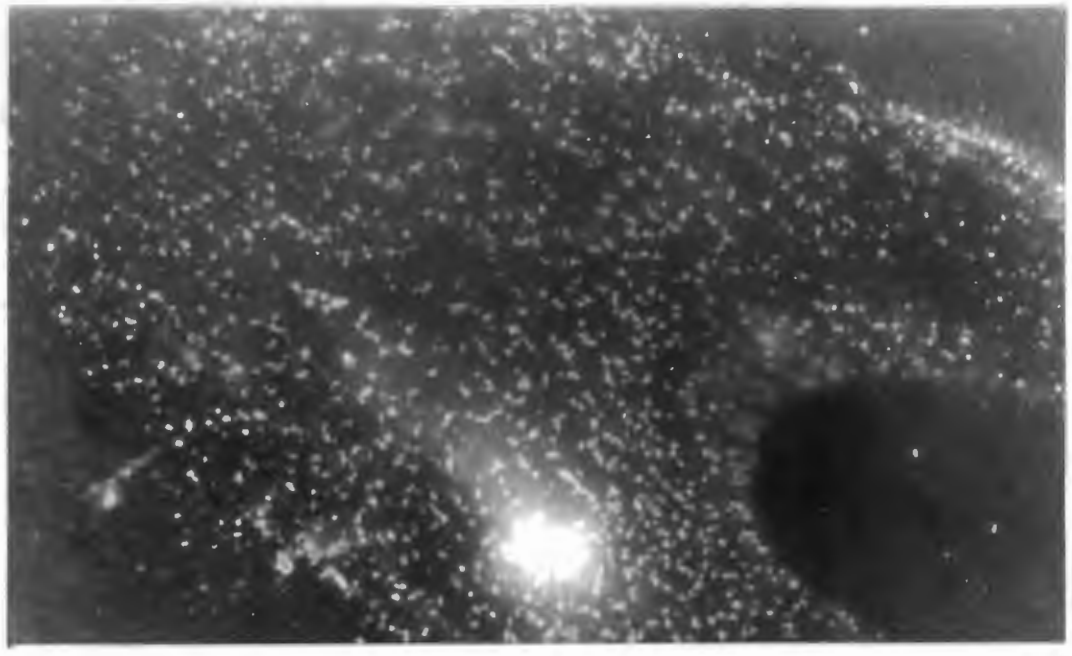


PLATE 3.3 C, D: LDL bound to the surface of GM 0203 normal human skin fibroblast cells at $37^{o}C$. LDL receptors have been probed by indirect immunocytochemistry (using LDL, RAAB, and FITC-GAR) and then detected by immunofluorescence in the light microscope. A fine, focal distribution of punctate LDL-specific fluorescence similar to that seen at $4^{o}C$ is seen in this micrograph. **D:** The LDL receptors appear to be arranged in linear arrays. Magnifications C, D = 1 640x.

Plate 3.4 A, B

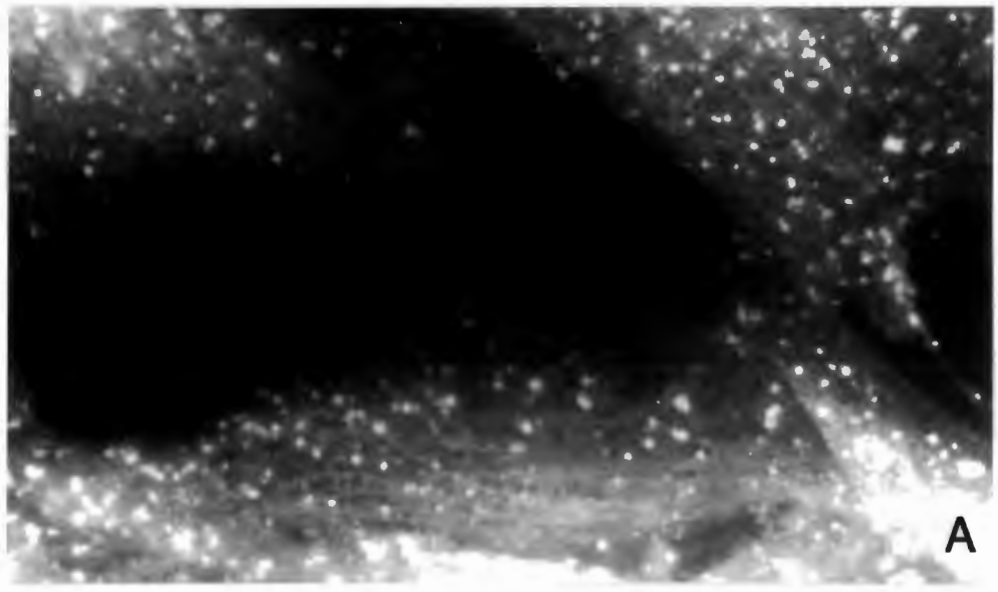


PLATE 3.4: Normal fibroblasts, GM 0203, incubated with LDL at 37°C, permeabilised with Triton X-100, and then prepared for immunofluorescence microscopy using RAAB and FITC-GAR. Note the intracellular fluorescent foci representing endocytosed LDL. They are larger than the surface foci seen in PLATE 3.3 A, C and D.
A: Fluorescence micrograph. **B**: Phase contrast micrograph of the same field as shown in A. Magnifications A, B = 1 480x.

Plate 3.5 A, B

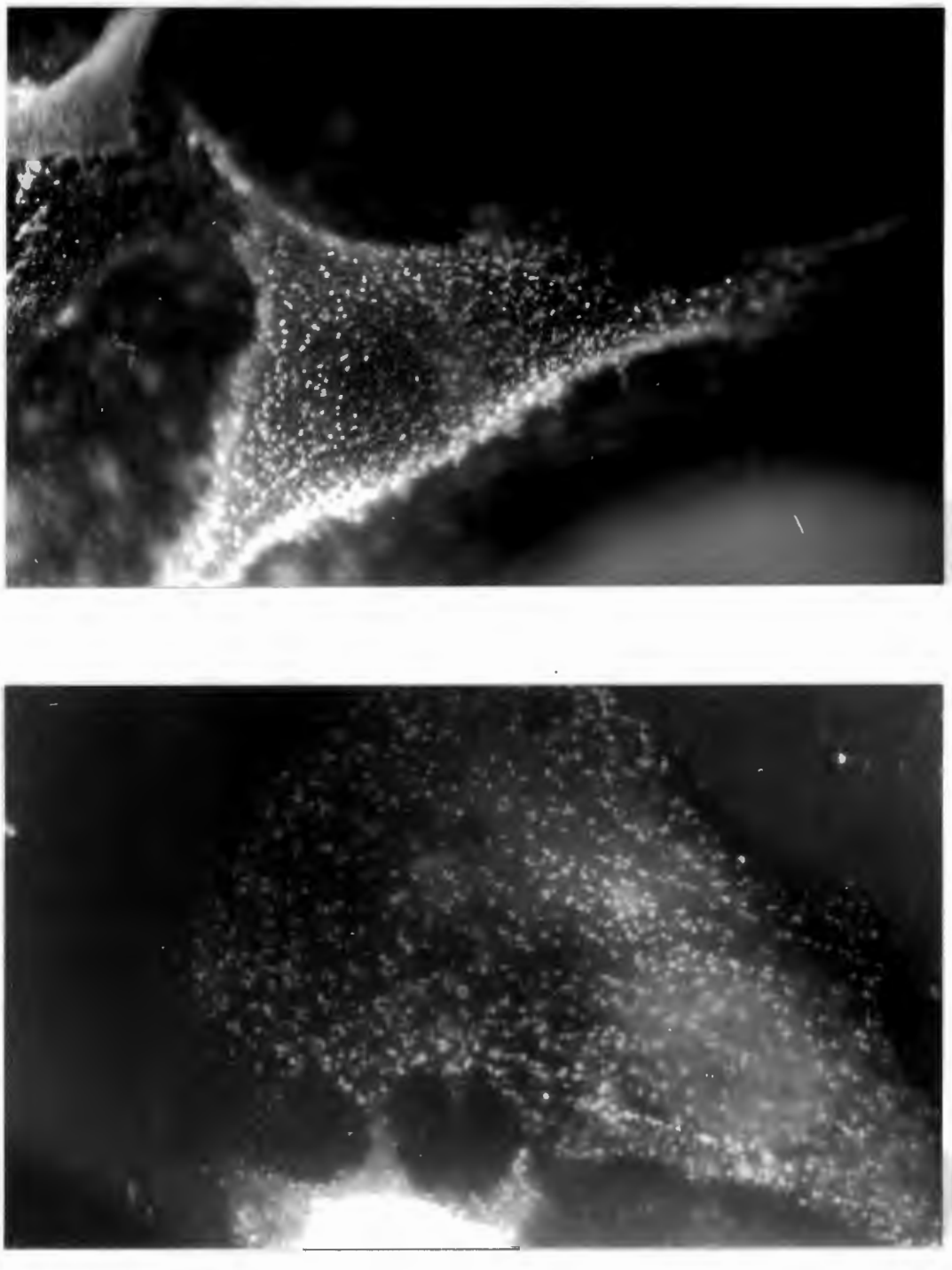
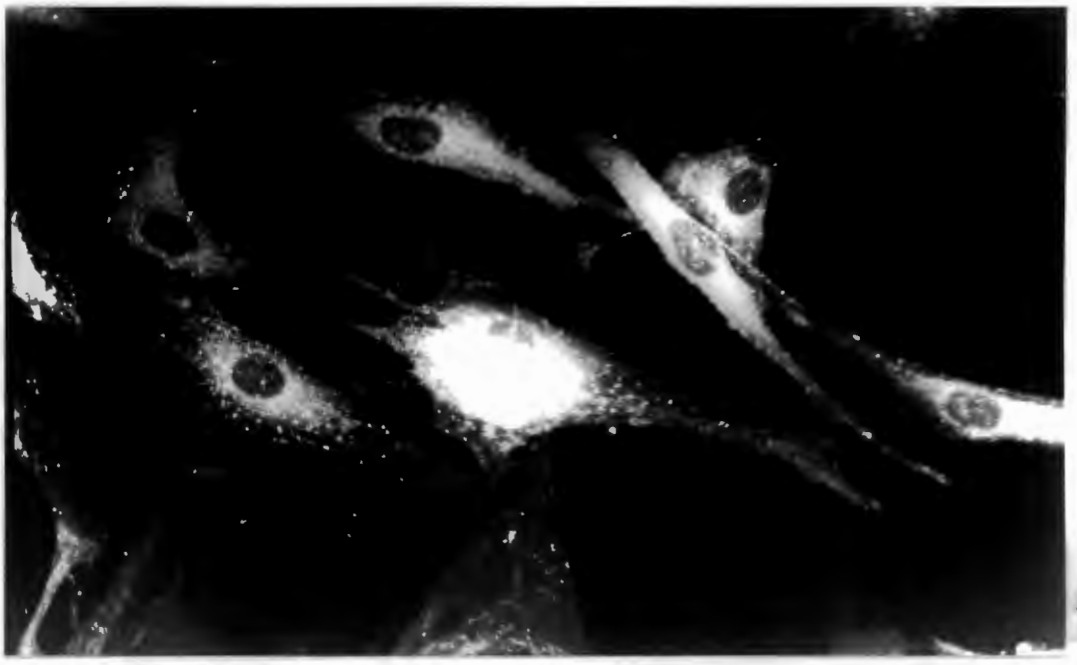


PLATE 3.5: Similar surface fluorescence patterns were obtained on normal fibroblasts when the LDL receptor was probed either with DiI-LDL or native LDL. **A**: Normal fibroblasts, GM 0203, incubated with DiI-LDL at 4°C. Direct fluorescence microscopy. Magnification = 980x. **B**: GM 0203, incubated with LDL at 4°C and detected by indirect immunofluorescence microscopy. Magnification = 1 680x.

Plate 3.6 A, B



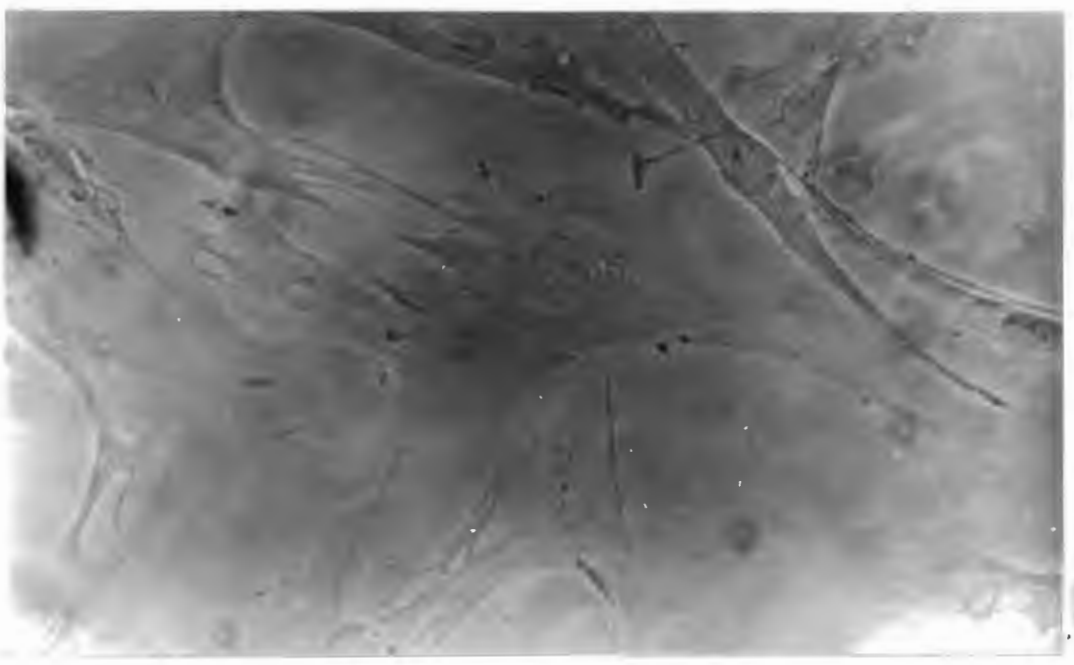
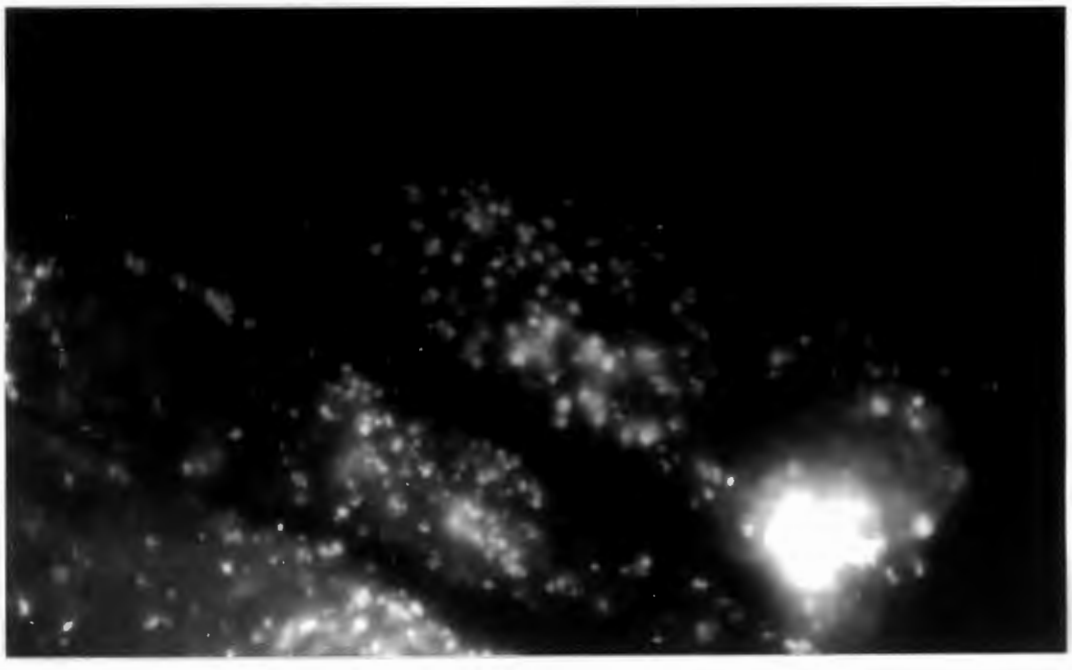
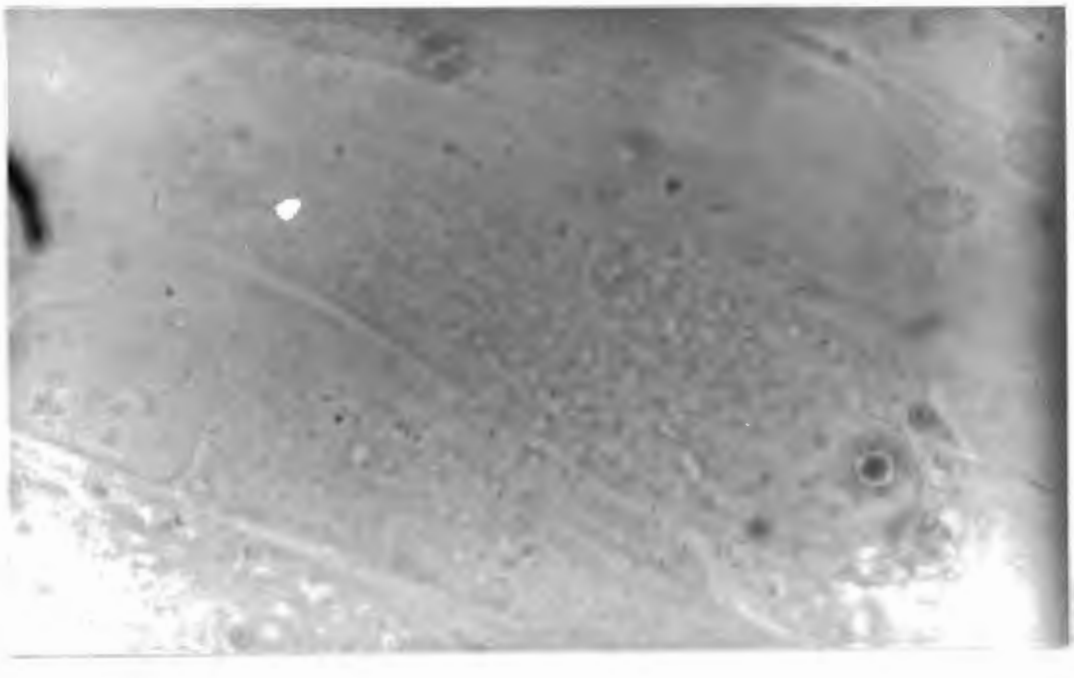


PLATE 3.6:
A: Normal fibroblasts, GM 3348, incubated with DiI-LDL at 37°C for 4 hours. Direct fluorescence microscopy.
B: Phase contrast micrograph of the same field as shown in A. Magnifications A, B = 388x.

Plate 3.6 C, D



C



D

PLATE 3.6:
C: Normal fibroblasts, GM 3348, incubated with DiI-LDL at 37^{o}C for 4 hours. Direct fluorescence microscopy.
D: Phase contrast micrograph of the same field as shown in PLATE 3.6 A. Magnifications C, D = 1 520x.

Interaction of LDL with Subconfluent and Postconfluent ECs

Surface Binding

The binding of DiI-LDL to upregulated, subconfluent ECs at 4°C was visualised by direct fluorescence microscopy (DFM) . DiI-LDL was distributed randomly all over the surfaces of the cells as fine punctate foci of intense fluorescence (PLATE 3.7 A-D) The foci appeared similar in size to those seen on the surfaces of human skin fibroblast controls (PLATE 3.8 A, B). No fluorescence was seen when contact-inhibited postconfluent cultures were incubated with DiI-LDL at 4°C (PLATE 3.9).

In order to check the pattern of surface-binding of DiI-LDL against another system, native LDL was incubated with ECs under similar conditions and its distribution visualized using indirect immunofluorescence techniques as outlined in MATERIALS and METHODS (p.71). Binding patterns similar to those seen with DiI-LDL were revealed on both subconfluent (PLATE 3.10 A, B) and postconfluent cells (PLATE 3.10 C). The subconfluent E8 EC surfaces (PLATE 3.10 A,B) are labelled with faint punctate foci resembling those seen on similarly labelled fibroblasts (PLATE 3.3 A, C, D) both in size and linear arrangement. The outline of cells in the postconfluent monolayer (PLATE 3.10 C) can be seen by the non-specific fluorescence on the cell margins. The cell surfaces are not fluorescently labelled indicating the absence of bound LDL. Note that PLATES 3.10 A,B and C have a background fluorescence which is partially attributable to non-specific binding of the fluorescent secondary antibody, GAR-FITC. Reference to PLATE 3.11 (subconfluent ECs without RAAB but with GAR-FITC) will clarify this.

In addition, part of the general background fluorescence encountered with this system of indirect immunofluorescence, was due to the non-specific binding of the primary antibody to the cell surfaces. Plate 3.12 shows a subconfluent E3 preparation which has received all components of the immunocytochemical procedure excluding LDL. In this control preparation no punctate fluorescent foci are visible because LDL was not bound, but a diffuse fluorescent background remains.

The practice of of pre-incubating the secondary antibody against cells to adsorb out the antibodies which might bind non-specifically, was attempted (MATERIALS and METHODS). In addition to reduced non-specific fluorescence, such adsorbed antisera gave poor specific fluorescence signals, when used later in experiments.

The standard set of controls for indirect immunofluorescence experiments, mentioned in MATERIALS and METHODS (pp. 71-72). was employed during every experiment.

To check for temperature-induced redistribution of surface receptors, subconfluent ECs were incubated with LDL at 37°C, a temperature favourable for receptor mobility in the plane of the membrane.[185, 15] DiI-LDL would have been endocytosed at 37°C and its intracellular fluorescence would have confused the interpretation of surface labelling patterns. Therefore indirect immunofluorescence microscopy (IIFM) was mandatory. LDL endocytosed at 37°C was inaccessible to the primary antibody,[8, 2] hence only surface LDL was detected. The distribution of surface-bound LDL at 37°C (PLATE 3.13) was similar to that seen at 4°C.

Plate 3.7 A - C

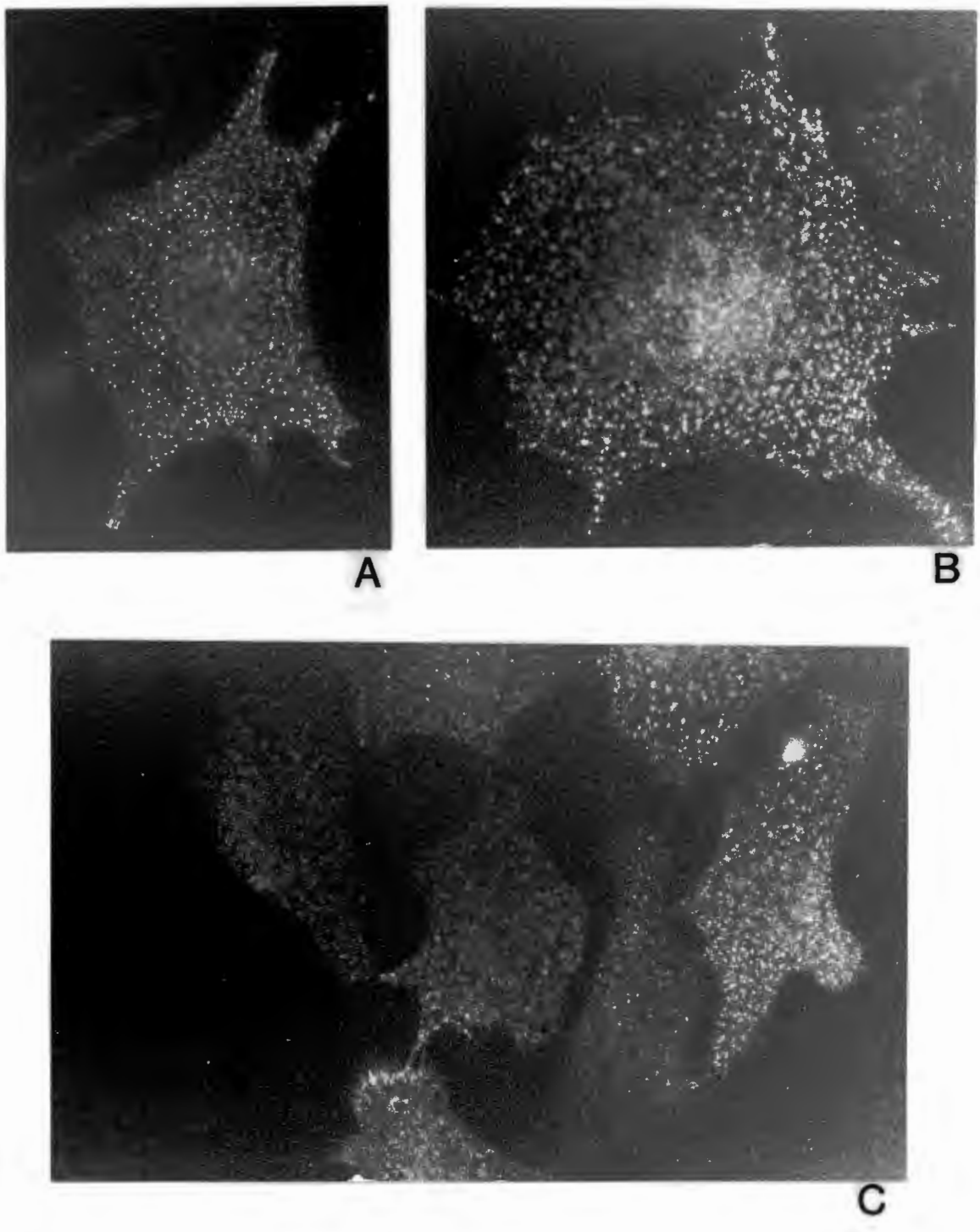


PLATE 3.7 A - C: Panel of plates depicting subconfluent E8 cells incubated with DiI-LDL (20µg/ml) at 4°C. The DiI-LDL has been bound in bright punctate foci randomly over the entire cell surface. The hazy central areas are nuclear bulges whose upper surfaces are not in focus (A, B) Magnifications: A = 640x, B = 1008x, C = 640x.

Plate 3.8 A, B

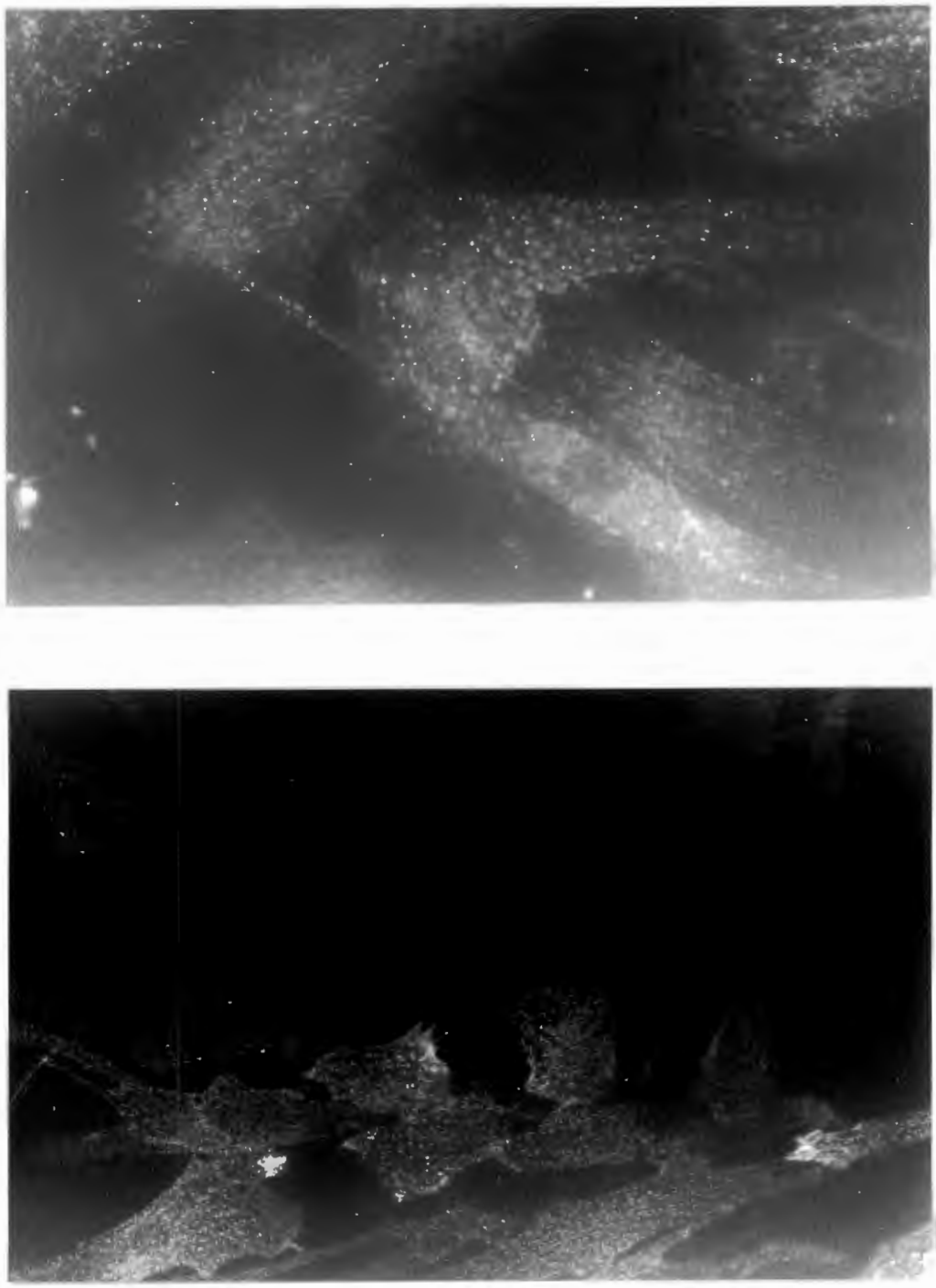


PLATE 3.8 A,B: GM 3348 fibroblasts incubated with DiI-LDL (20µg/ml) at 4^{o}C. The DiI-LDL has been bound in bright punctate foci over the entire cell surface. The fluorescent foci are sometimes arranged in linear arrays along the long axis of the cells. Magnifications: A = 640x, B = 400x.

Plate 3.9 A-D

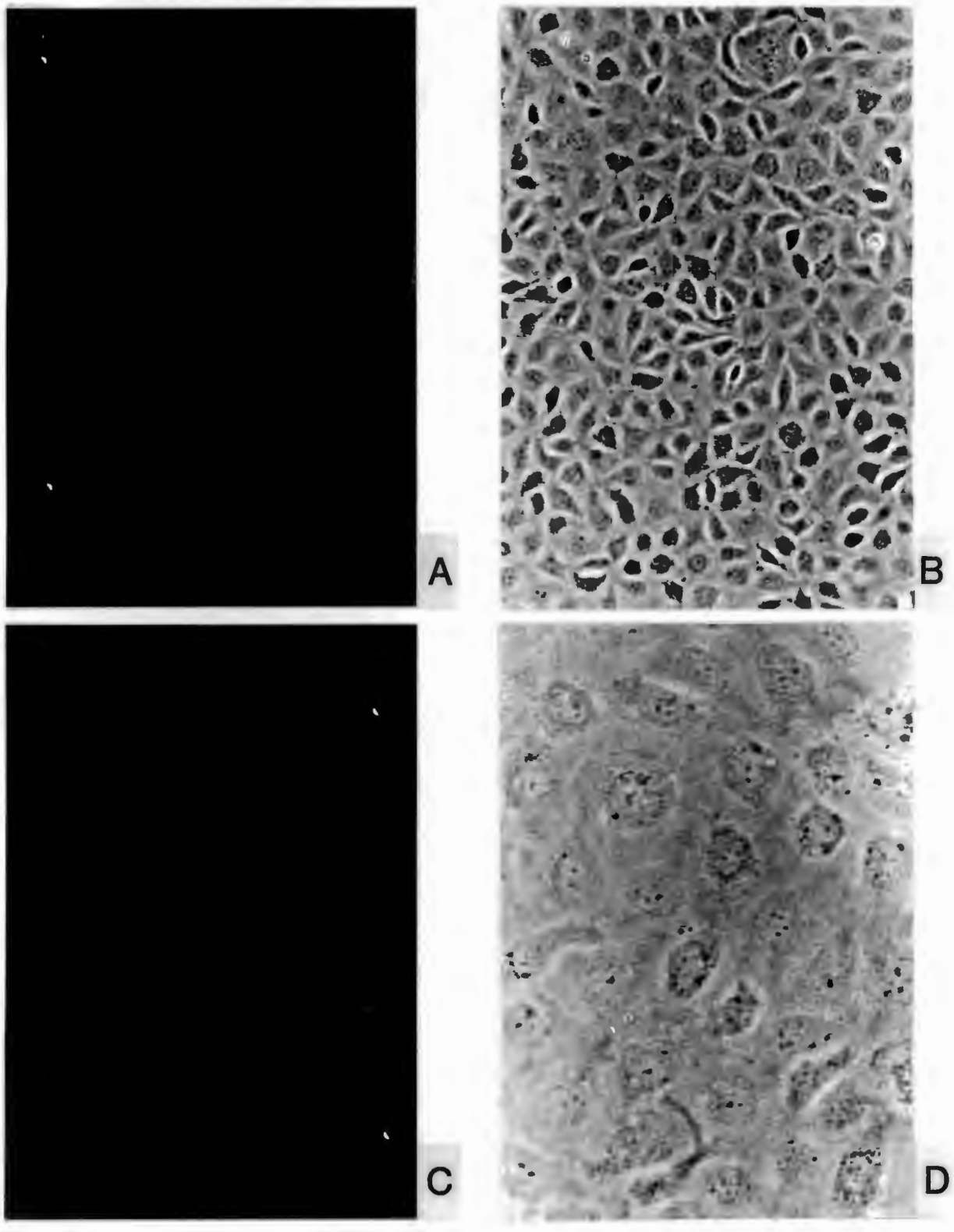
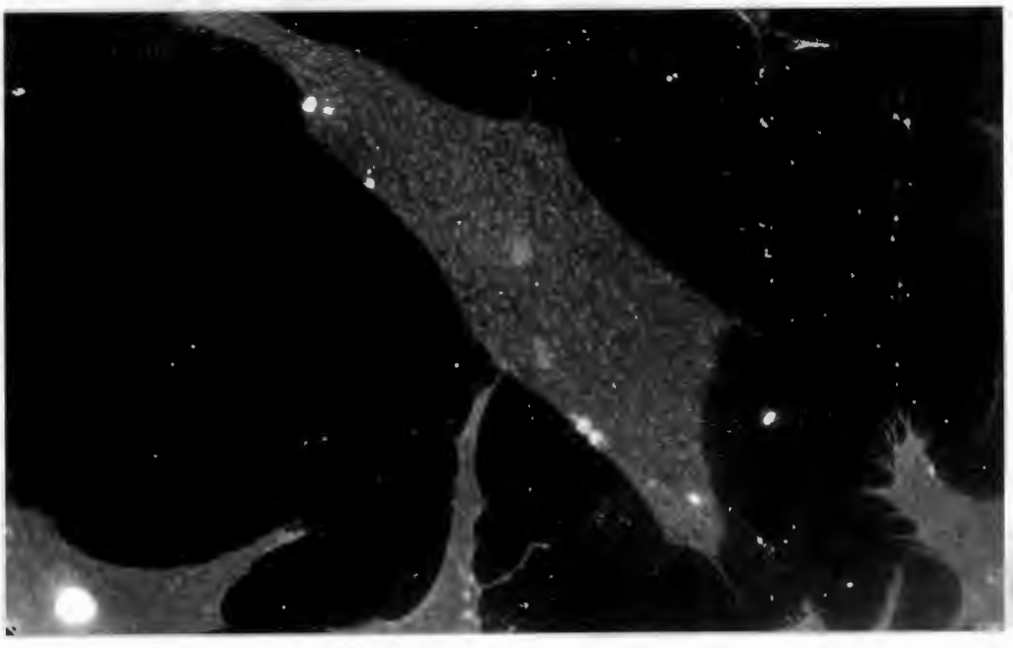
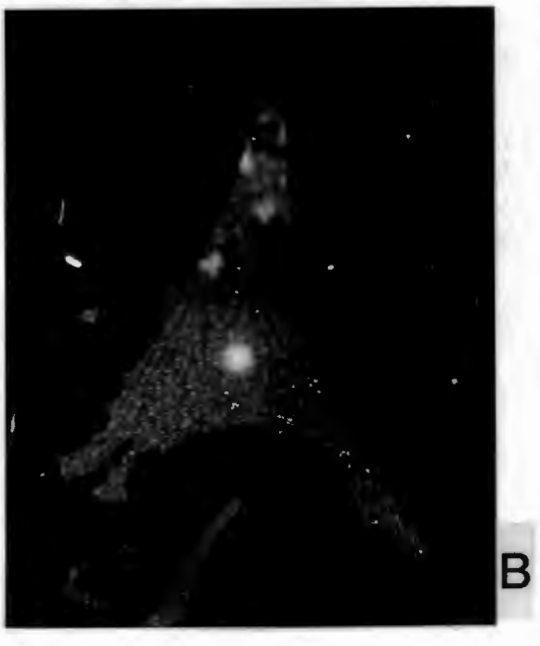


PLATE 3.9:
A, C: Direct fluorescence micrographs of postconfluent E8 cells incubated with DiI-LDL (20µg/ml) at 4°C. The almost total absence of fluorescence indicates that binding of DiI-LDL was virtually nil. **B, D:** Phase contrast micrographs of the same fields as shown in A and C respectively. Magnifications: A, B = 160x; C, D = 370x.

Plate 3.10 A-C





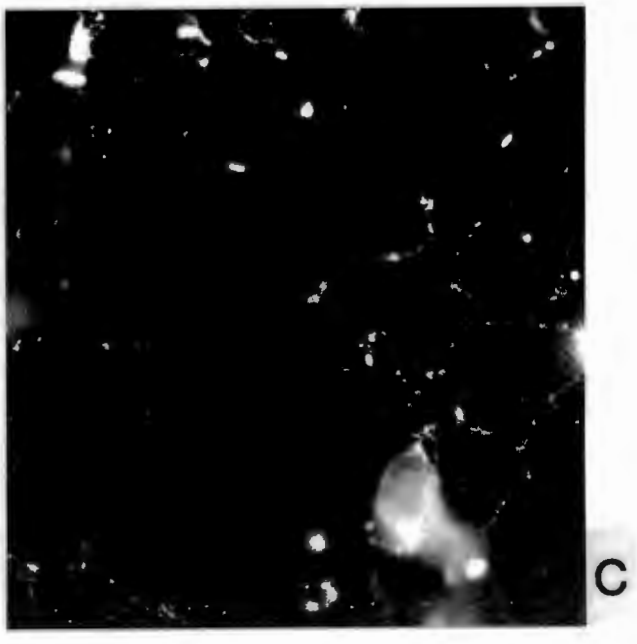


PLATE 3.10:
A, B: Indirect immunofluorescence micrographs of subconfluent E8 cells incubated with LDL (10μg/ml) at 4°C The fluorescence pattern is reminiscent of that seen on fibroblasts similarly labelled. Note the linear arrays of fluorescent foci.
C: Postconfluent E8 labelled as in A and B. The cell margins have non-specific fluorescence. But the cell surfaces are devoid of LDL-specific fluorescence. Magnifications: A, B = 640x, C = 580x.

Plates 3.11 and 3.12

PLATE 3.11: Indirect immunofluorescence micrographs of subconfluent E8 cells incubated with LDL (20µg/ml) at 4°C. The primary antibody (RAAB) was omitted from the immunocytochemical procedure, but the secondary antibody, FITC-GAR, was included. Note the non-specific background fluorescence. Magnification = 1 600x.

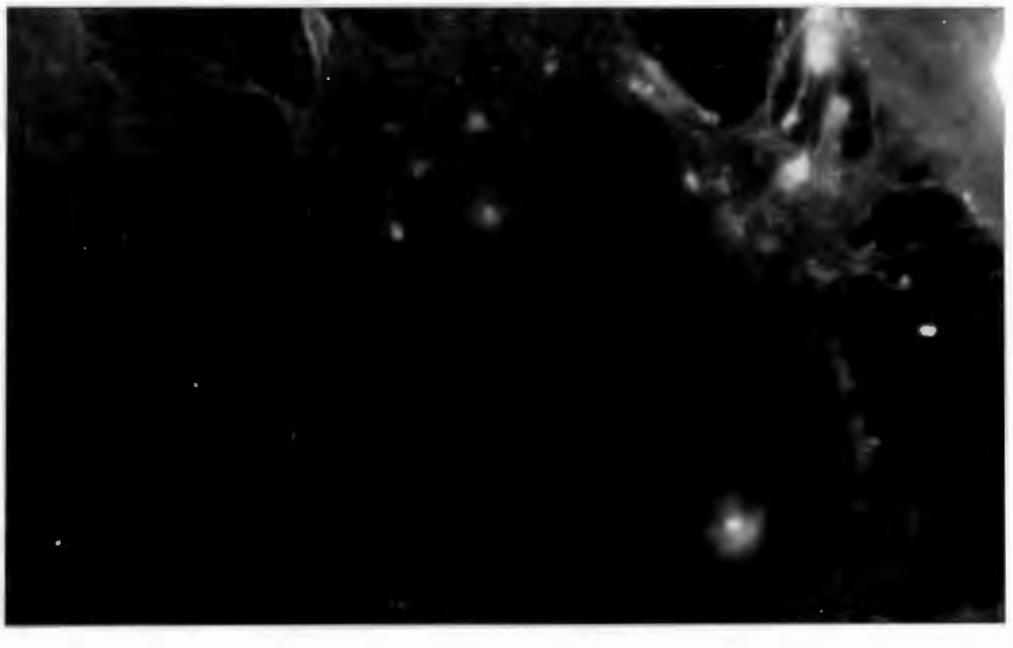


PLATE 3.12: Indirect immunofluorescence micrographs of subconfluent E3 cells incubated with all the components of the immunocytochemical process except the ligand, LDL, which has been omitted. Note the diffuse, non-specific background fluorescence. Magnification = 1 680x.

Plate 3.13

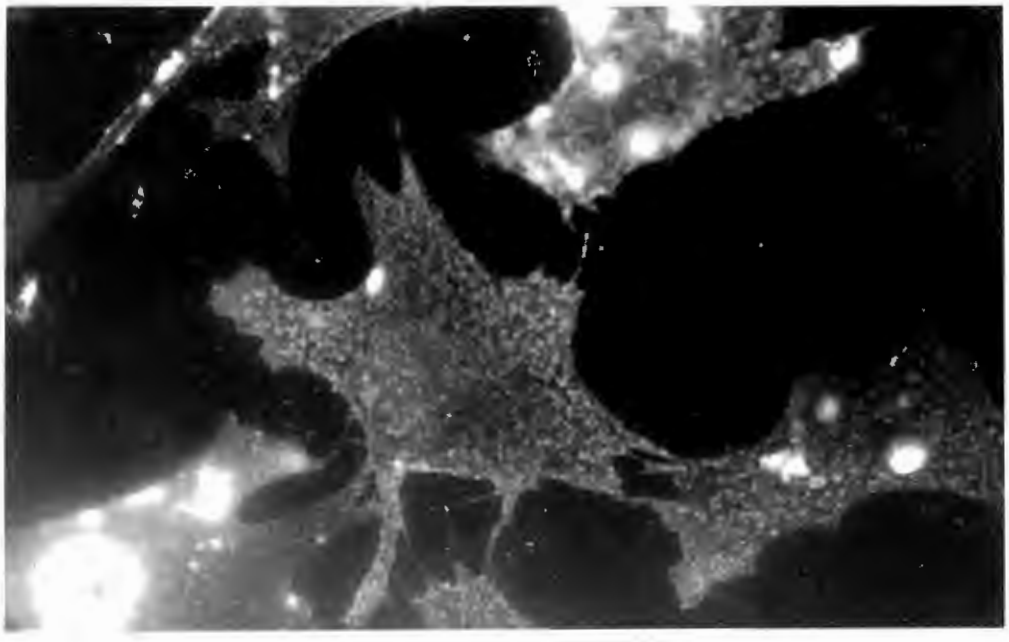


PLATE 3.13: Indirect immunofluorescence micrograph of subconfluent E8 cells incubated with LDL (20µg/ml) at 37°C. The surface distribution of LDL is similar to that seen at 4°C. Magnification = 640x.

Intracellular Localisation of LDL

Intracellular LDL in subconfluent ECs incubated with LDL at 37°C was detected by indirect immunofluorescence after detergent permeabilization of the membranes with Triton X-100 (MATERIALS and METHODS). PLATE 3.14 shows the immunoreactive material to be organised into relatively large round foci. This represents only intracellular ligand because the cells were treated with dextran sulphate (10 mg/ml in PBS for 1 h at 4°C) to release extracellular LDL before immunocytochemistry. Similar results were obtained with DiI-LDL (PLATE 3.15).

Subconfluent E8 cells were incubated with DiI-LDL for 4 hours at 37°C. Surface-bound DiI-LDL was removed by dextran sulphate treatment as above. The DiI label accumulated intracellularly and became intensely concentrated in the perinuclear region. Numerous large fluorescent foci appeared in the peripheral cytoplasm. Tendrilous elements in the perinuclear region were also labelled with DiI after the prolonged incubation with DiI-LDL.

When DiI-LDL was incubated with postconfluent E8 cells at 20µg/ml at 37°C the uptake of DiI-LDL as evidenced by fluorescence was too low to be recorded photographically (PLATE 3.16 A,B).

Competition Studies

Unlabelled LDL was allowed to compete with DiI-labelled LDL for surface receptors at 4°C and 37°C in subconfluent EC cultures. Excess unlabelled LDL (300µg/ml) markedly reduced the binding and uptake of fluorescent DiI-LDL (PLATE 3.17). A similar decrease in the intracellular DiI-LDL content of ECs incubated at 37°C with DiI-LDL in the presence of excess native LDL was also noted (data not shown). This competition between LDL and DiI-LDL for receptors shown by fluorescence microscopy could also be demonstrated quantitatively by fluorimetric determinations of DiI in competitive uptake studies using DiI-LDL. (see Fluorimetric Determination of Uptake of DiI-Labelled LDL, p.70). The fluorimetric technique was not sensitive enough to assay surface binding, so the data reflects bound plus intracellular DiI-LDL after a 4 hour incubation at 37°C. After endocytosis of DiI-LDL, the DiI moeity remains in the cell dissolved into membranes. It is not released into the medium after degradation of the LDL, hence the intracellular level reflects accumulated uptake.[204] A 10-fold excess of LDL (300µg/ml) decreased the uptake of DiI-LDL (at 50µg/ml) to 2100 ng DiI/mg cell protein/4 hr from an initial 4000 ng DiI/mg cell protein/4 hr in upregulated E8 subconfluent cells. This was evidence of competition for saturable high-affinity receptors responsible for about 50% of the total uptake.

In a parallel experiment using ^{125}I-LDL at 50µg/ml cells took up a total of 8723 which dropped to 2071 ng LDL protein/mg cell protein/4hr (bound + intracellular + degraded LDL). Therefore 76% of the total uptake was by high-affinity receptors. The concentrations of the ligands were the same in both experiments so it appears that although DiI-LDL does bind to high-affinity receptors for which LDL will compete, ^{125}I-LDL is a more efficient probe for LDL receptors. The efficiency of uptake of the radiolabelled ligand was twice that of the fluorescent one on the basis of LDL protein taken up.

Pulse-Chase Experiments

Pulse-chase experiments were performed to determine the fate of endocytosed LDL after 30 minutes at 37°C. This period of time was chosen because very little degradation occurred within 30 minutes of the initiation of endocytosis. E8 cells were treated with DiI-LDL at 20µg/ml in MEM-LPDS at 4°C for 2 hours (pulse). The cells had been pre-incubated in MEM-LPDS for 48 hours. After washing in PBS to remove unbound material, they were incubated at 37°C for 30 minutes (chase) in MEM-LPDS. By direct fluorescence microscopy, DiI was seen to have accumulated in the perinuclear regions of the cytoplasm in discrete round foci (PLATE 3.18). This fluorescence represented uptake of surface-bound ligand and its accumulation in perinuclear vesicles. Comparing this with an image of an E8 cell exposed to DiI-LDL continuously at 37°C (PLATE 3.19) for 1 hour, the latter has a population of peripheral, smaller foci in addition to the larger perinuclear vesicles. The two populations are clearly separated by a zone relatively free of vesicles. The cells were treated with dextran sulphate to remove the surface-bound DiI-LDL.

Clearly, the DiI label moves from peripheral sites to perinuclear sites with time and accumulates there in larger foci. It is likely that peripheral foci represent endosomes and perinuclear foci fused endosomes perhaps in the early stages of lysosomal fusion.

Plate 3.14 A,B

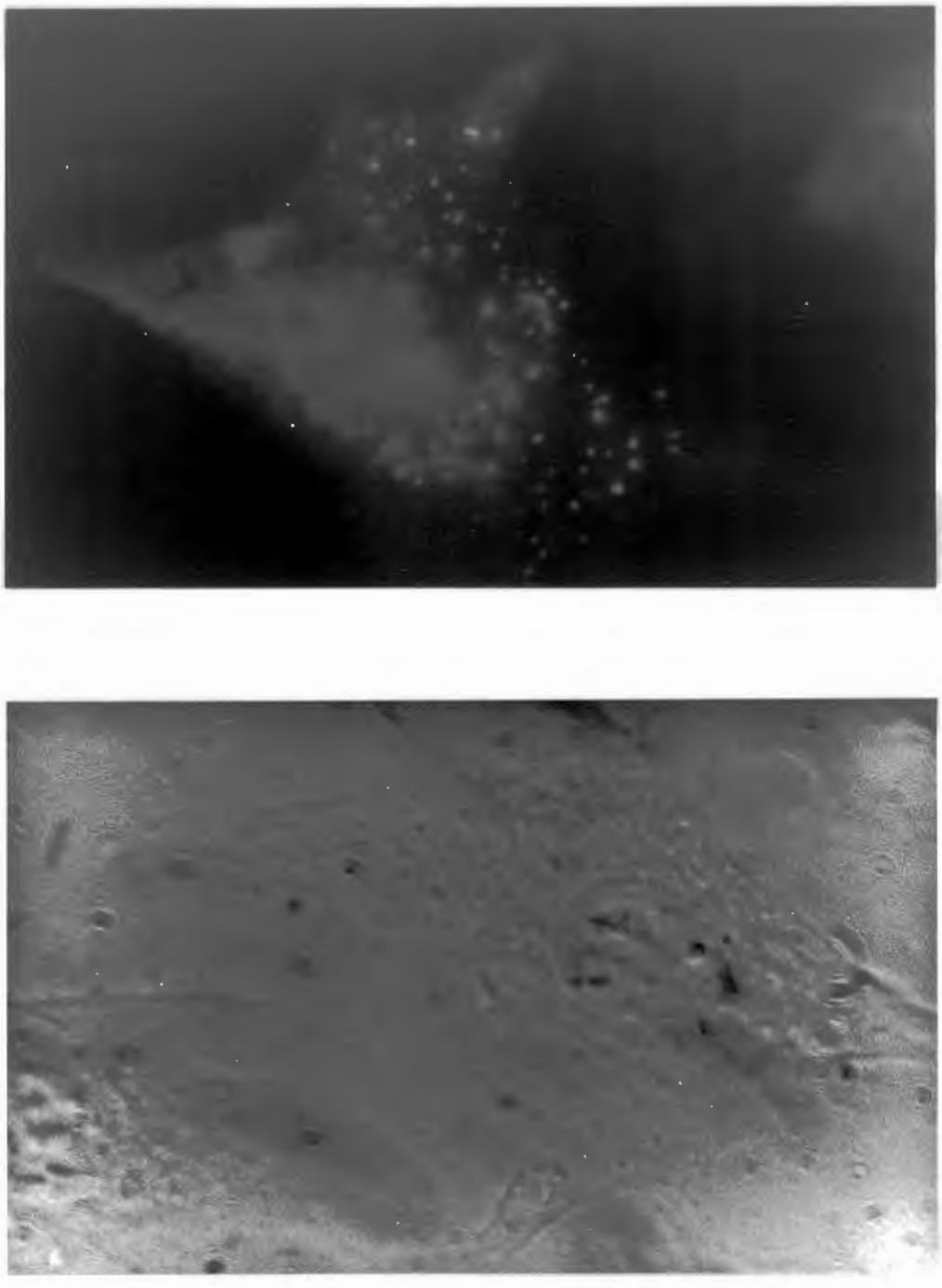


PLATE 3.14: Indirect immunofluorescence micrograph of subconfluent E3 cells incubated with LDL (20µg/ml) at 37°C, fixed and then treated with Triton X-100 to permeabilise the cells to the antibodies used for immunocytochemistry. The surface-bound LDL has been removed by dextran sulphate, so that only intracellular LDL has been detected. Note the relatively large foci presumably representing fused endosomes or lysosomes. **A**: Fluorescence micrograph. **B**: Phase contrast micrograph of the same field as shown in A. Magnifications A, B = 660x.

Plate 3.15

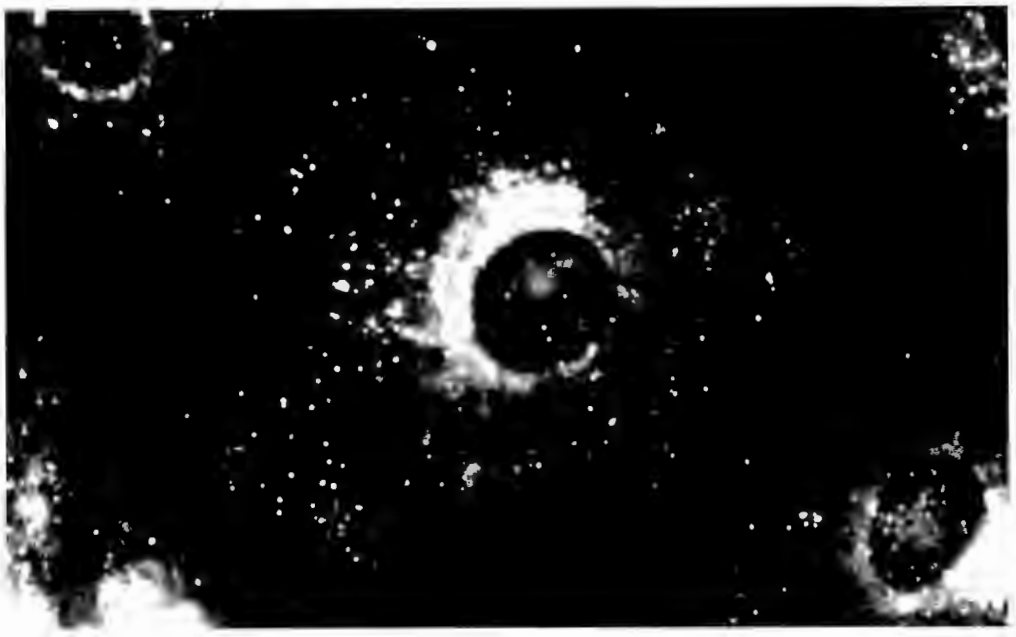
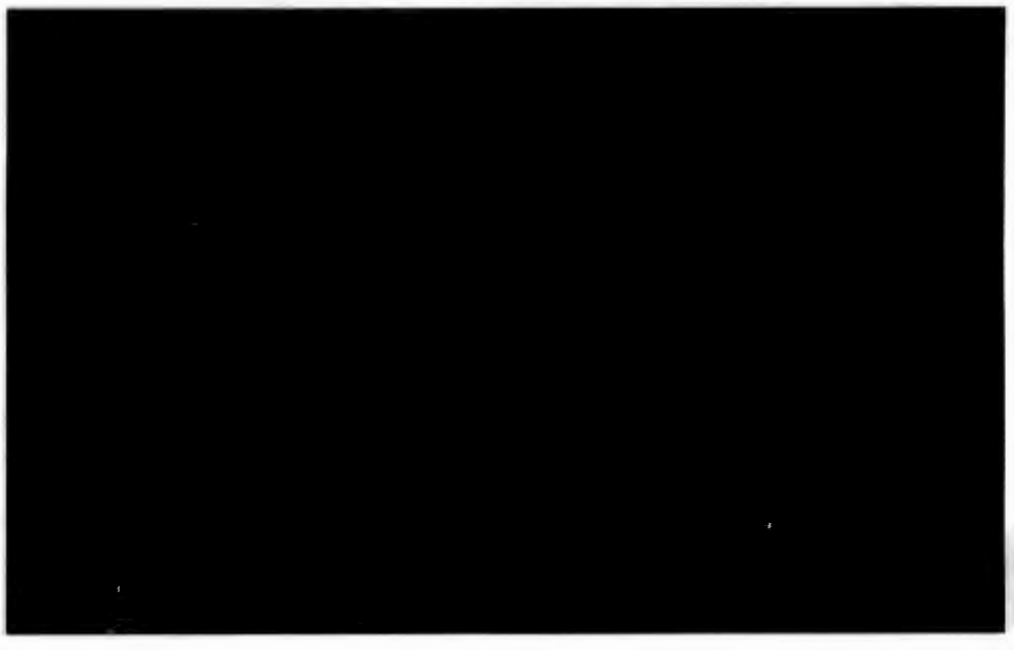


PLATE 3.15: E8 cells incubated with DiI-LDL (20µg/ml) at 37°C for 4 h. The surface-bound DiI-LDL has been removed by dextran sulphate, so that only intracellular DiI-LDL has been detected. Note the large foci presumably representing fused endosomes or lysosomes; and the extremely dense accumulation of DiI in the regions.
Direct fluorescence micrograph.
Magnification = 640x.

Plate 3.16 A, B



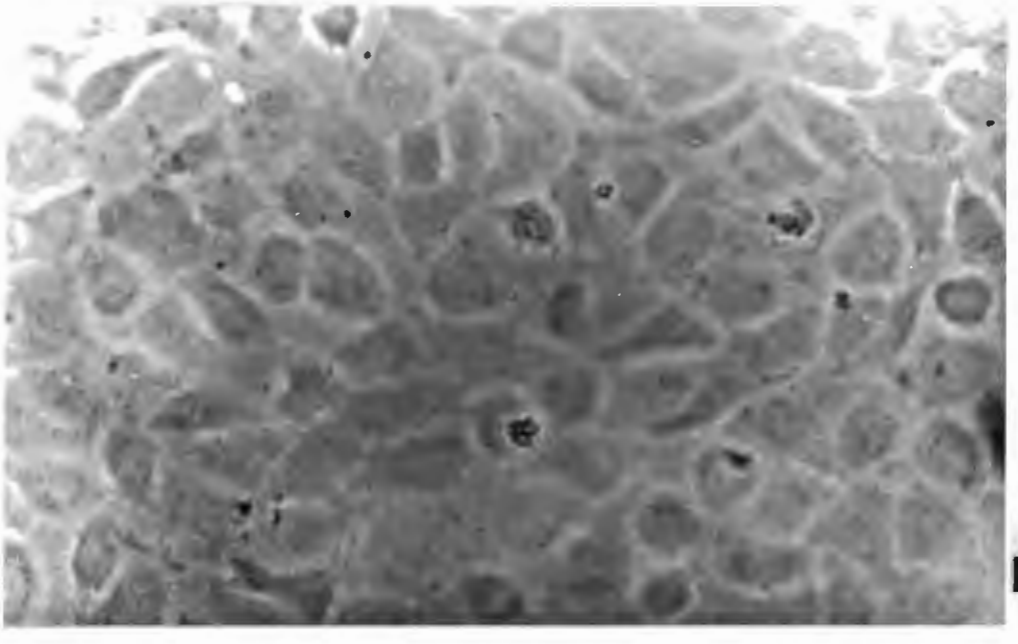


PLATE 3.16: Direct fluorescence micrograph of postconfluent E8 cells incubated with LDL (20µg/ml) at 37°C for 4h. DiI fluorescence was too low to be recorded photographically. **A**: Fluorescence micrograph. **B**: Phase contrast micrograph of the same field as shown in A Magnifications A, B = 380x.

Plate 3.17

PLATE 3.17:
Decreased binding of DiI-LDL at $4^{o}C$ to subconfluent E8 cells co-incubated with excess unlabelled LDL (300µg/ml).
This demonstrates competition between the native and the DiI-LDL for LDL receptors. Magnification = 1 600x.

Plate 3.18 A,B

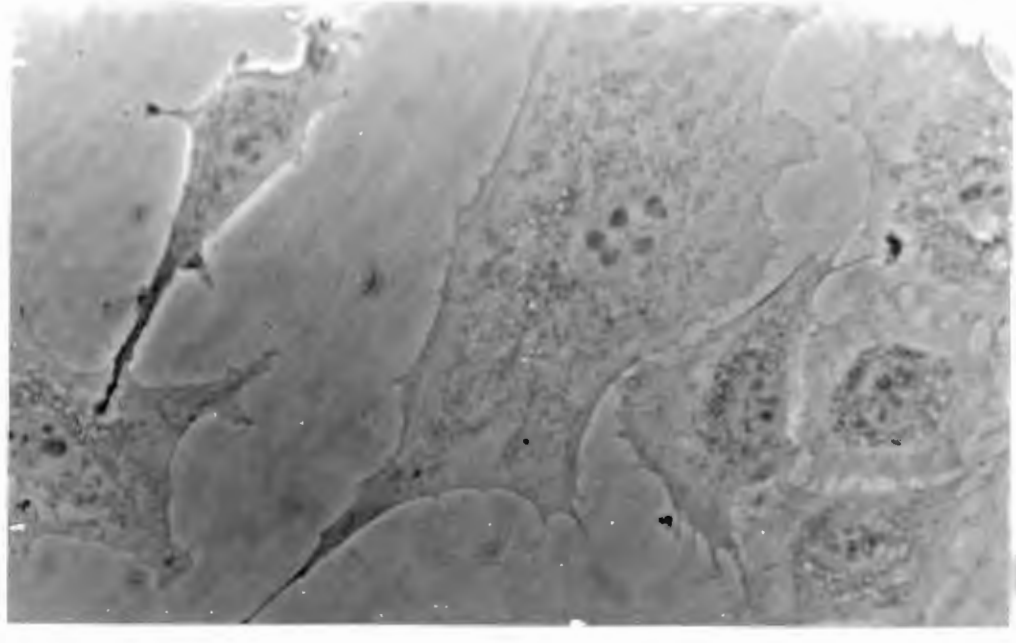


PLATE 3.18: Direct fluorescence micrograph of subconfluent E8 cells incubated with DiI-LDL (20µg/ml) at 4°C for 60 minutes (pulse), washed and then incubated at 37°C for 30 minutes (chase) before fixation. **A**: Fluorescence micrograph showing the perinuclear accumulation of vesicles, probably prelysosomal. The position within the cells can be deduced by comparison with **B**: the phase contrast image of the same field. Magnifications A, B = 560x.

Plate 3.19

PLATE 3.19:
Fluorescence micrograph of an E8 cell from a subconfluent culture exposed to DiI-LDL continuously at 37°C for 1 hour. There are at least two size-populations of fluorescent foci, which most probably represent endosomes (smaller foci seen at the cell periphery) and fused endosomes in the CURL (larger juxtanuclear foci). Note the relatively clear zone separating the two populations. The cells were treated with dextran sulphate to remove the surface-bound DiI-LDL. Magnification = 1 640x.

AcLDL Interactions with ECs

DiI-AcLDL bound to the surface of postconfluent E8 at 4°C as extremely fine punctate fluorescent foci randomly distributed over the entire cell surface as revealed by DFM (PLATE 3.20) Very fine foci representing discrete microdomains on the cell surface were clearly resolved. The foci are tightly packed when compared to the foci of surface-bound DiI-LDL (see PLATE 3.1).

AcLDL bound to the surface of postconfluentE8at 37°C was monitored by IIFM (PLATE 3.21). DiI-ACLDL would have been unsuitable as a selective surface probe because it would have been detected intracellularly by direct fluorescence. The distribution of fluorescent foci due to surface-bound AcLDL was similar to that seen at 4°C al though it was faint and difficult to record on micrographs (see Photomicrography, p.72) Heavy FITC labelling was also seen in the intercellular clefts but this was considered to be non-specific as it was also found in controls from which AcLDL was omitted.

Internalized DiI-AcLDL could be demonstrated by DFM in postconfluent
E8 cells which had been exposed to DiI-AcLDL at 20ug/ml continuously for 4 hours at 37°C (PLATE 3.22) In every cell, the DiI label accumulated in the perinuclear cytoplasm in such massive amounts that it often appeared as a continuous zone of fluorescence not easily resolvable into foci. Numerous fluorescent foci were also present in the peripheral cytoplasm.

Competition studies revealed that excess unlabelled AcLDL (200µg/ml) markedly depressed uptake of DiI-AcLDL (20µg/ml) as evidenced by diminished fluorescence (data not shown).

Pulse-chase experiments involving postconfluent ECs exposed to DiI-AcLDL resulted in intracellular DiI accumulations (PLATE 3.23) similar to those seen with DiI-LDL in subconfluent cultures (PLATE 3.18).

Plates 3.20 and 3.21

PLATE 3.20: Direct fluorescence micrograph of postconfluent E8 cells incubated with DiI-AcLDL (20µg/ml) at 4°C. The extremely fine punctate fluorescent foci randomly distributed over the entire cell surface represent microdomains to which the ligand has bound. All the fluorescence could be resolved into fine foci. Diffuse fluorescence is apparent in areas not in focus owing to the bulging profiles of the monolayered cells and the optical sectioning effect of the planapochromatic lens used. Magnification = 640x.

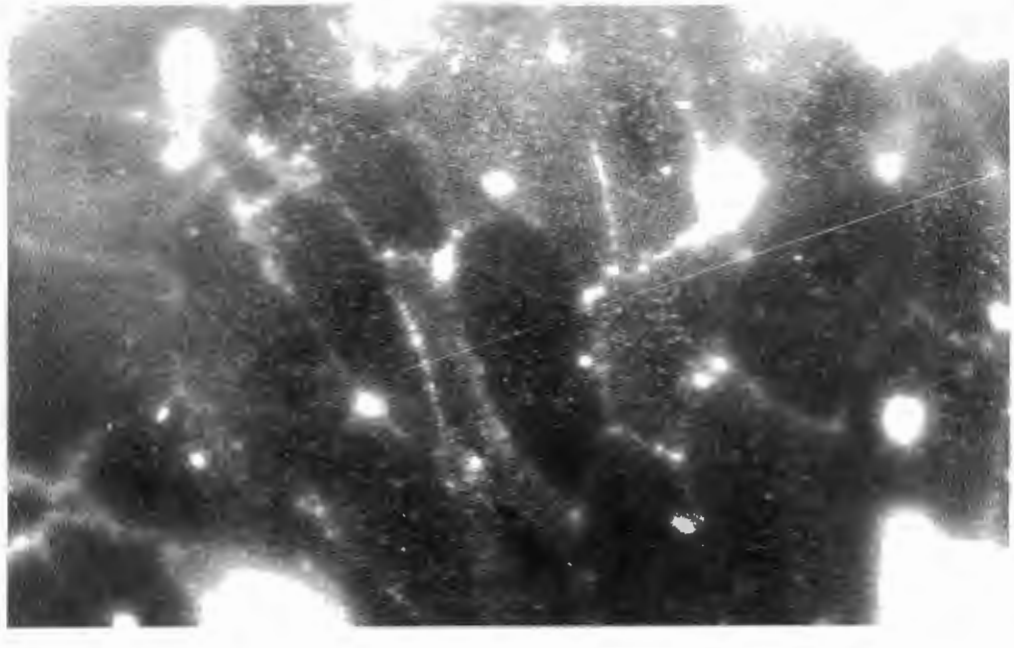


PLATE 3.21: Light micrograph of postconfluent E8 cells incubated with AcLDL at a concentration of 20µg/ml) at 4°C detected by indirect immunofluorescence. Fluorescent foci representing surface-bound AcLDL can be seen covering the surfaces of the cells. The fluorescence was faint and difficult to record photographically. Non-specific labelling of the intercellular clefts is also seen. Magnification = 930x.

Plate 3.22 A,B

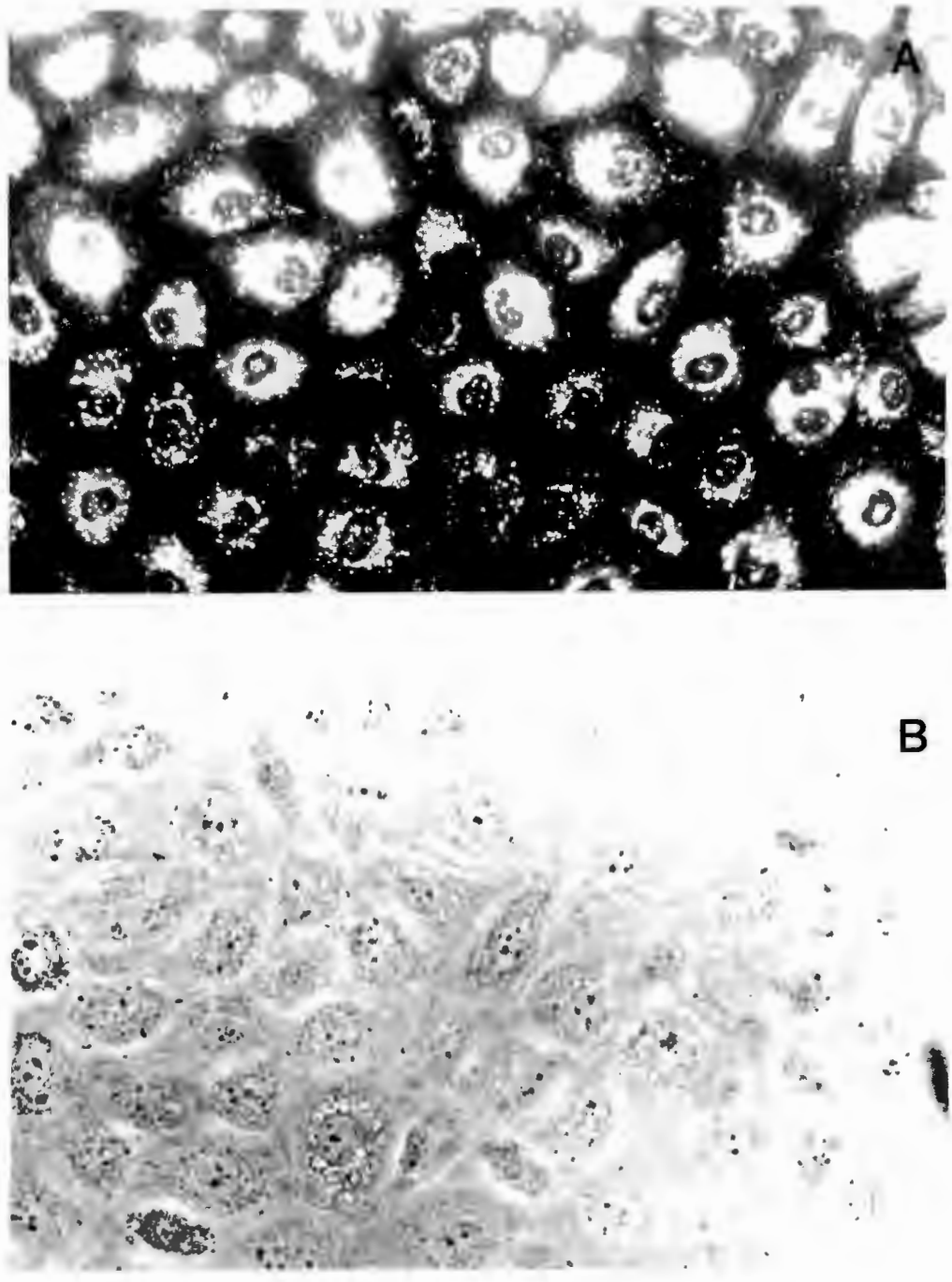


PLATE 3.22: Postconfluent E8 cells incubated with DiI-AcLDL at a concentration of 20µg/ml) at 37°C continuously for 4 h. DiI has accumulated in the cytoplasm as large bright fluorescent foci and in massive amounts in the perinuclear regions. **A**: Fluorescence micrograph. **B**: Phase contrast micrograph of the same field as shown in A Magnifications A, B = 400x.

Plate 3.23 A, B

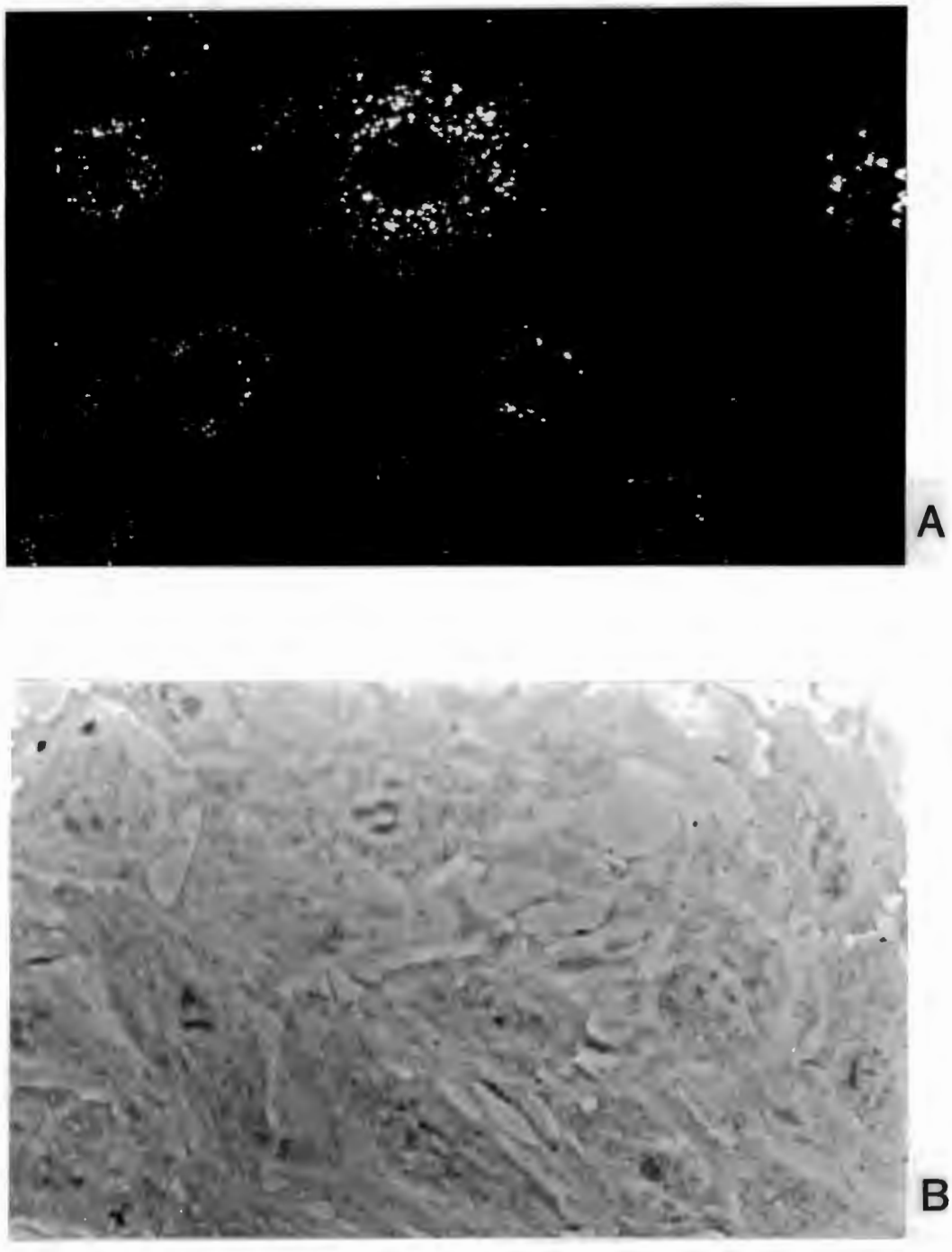


PLATE 3.23: Direct fluorescence micrograph of postconfluent E8 cells incubated with DiI-AcLDL (20µg/ml) at 4^{o}C for 60 minutes (pulse), washed and then incubated at 37^{o}C for 30 minutes (chase) before fixation. **A**: Fluorescence micrograph showing the perinuclear accumulation of large foci (probably fused endosomes just prior to fusion with lysosomes. The perinuclear position of the foci can be deduced by comparison with **B**: the phase contrast image of the same field Magnifications A, B = 420x.

Wounding Experiments

Confluent E8 cultures were wounded (Materials and Methods, p. 74) by denuding a narrow strip of plastic dish substratum of cells. Within 12-24 hours, cells were seen in the previously clear wound path (PLATE 3.24 A). Cells on the edge of the "wound" had elongated and appeared to be streaming into the clear area (PLATE 3.24 B). Regenerating wounded cultures incubated at $4^{o}C$ with DiI-LDL at 20µg/ml revealed punctate fluorescent foci on the surfaces of the active edge cells and isolated cells in the wound path, but not on cells in the confluent areas. Similar incubations, but at $37^{o}C$, revealed a marked contrast between the responses to the different probes by "edge" or active cells and by cells in the postconfluent areas. DiI-LDL labelled cells brilliantly on the edge of the wound (PLATE 3.25). This label was intracellular since dextran sulphate treatment of the cells did not diminish its intensity. Confluent areas of the same culture were not labelled by the DiI-LDL.

In sharp contrast, DiI-AcLDL labelled all the cells, regardless of their positions with respect to the wound (PLATE 3.26). DiI-AcLDL at 20µg/ml bound as tiny fluorescent foci on cell surfaces in areas of postconfluent cells and in wound areas. The pattern of DiI-AcLDL labelling took the form of large round fluorescent foci in the cytoplasm of all the cells in the regenerating culture (PLATE 3.27 A, B). The perinuclear regions were so highly fluorescent (PLATE 3.27 A), that they appeared as brilliant perinuclear coronas. In these cases, individual foci were difficult to resolve. PLATE 3.27 B shows a number of cells in most of which the focal nature of the perinuclear fluorescence can be seen.

Plate 3.24 A,B

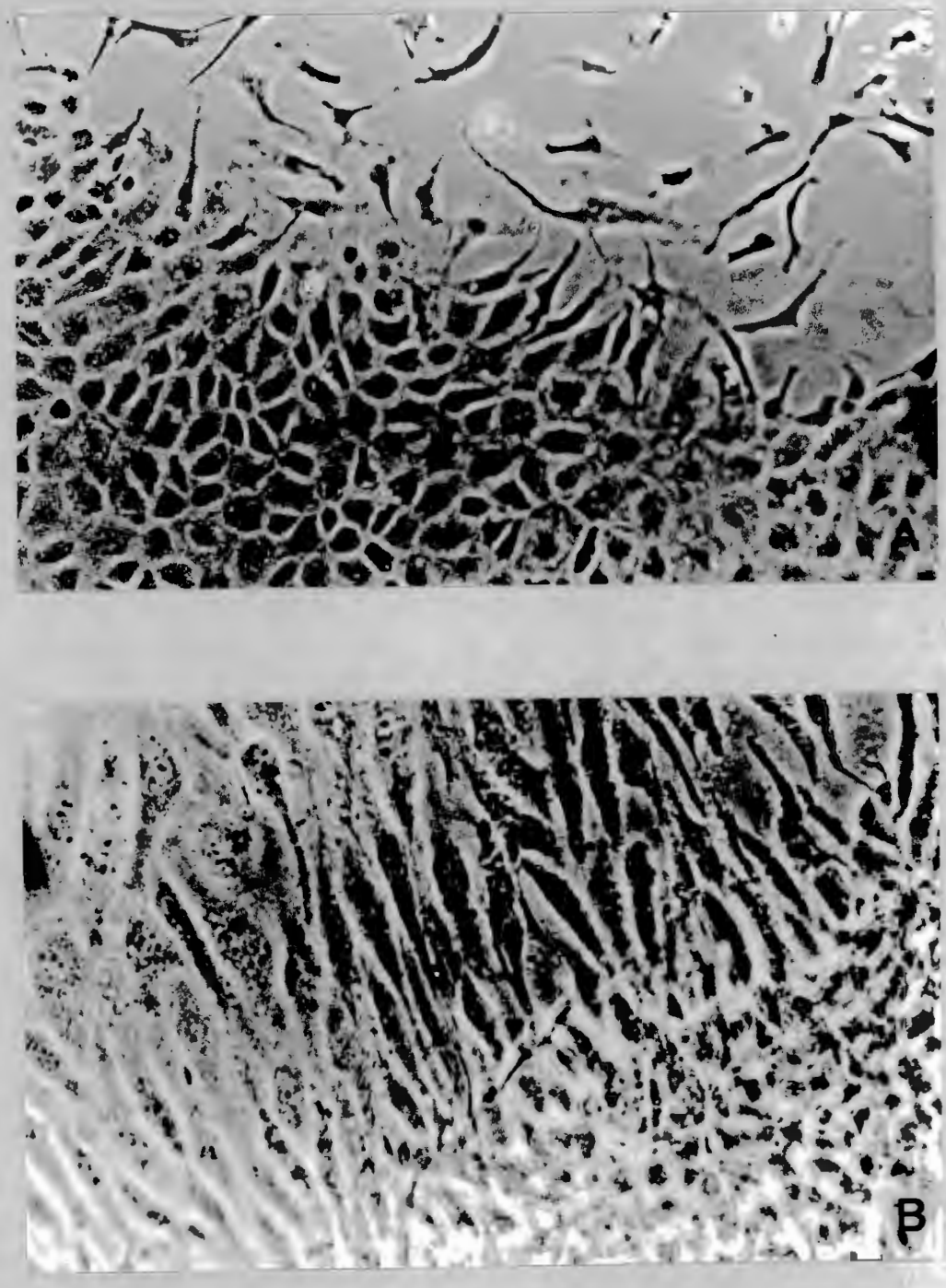


PLATE 3.24: Phase contrast micrograph showing the edge of a wounded postconfluent monolayer of E8 cells. **A**: After a regeneration period cells were seen to have migrated into the previously denuded wound path. **B**: Cells on the wound edge elongated and streamed into the denuded areas.
Magnifications: A = 176x, B = 408x.

Plate 3.25 A,B

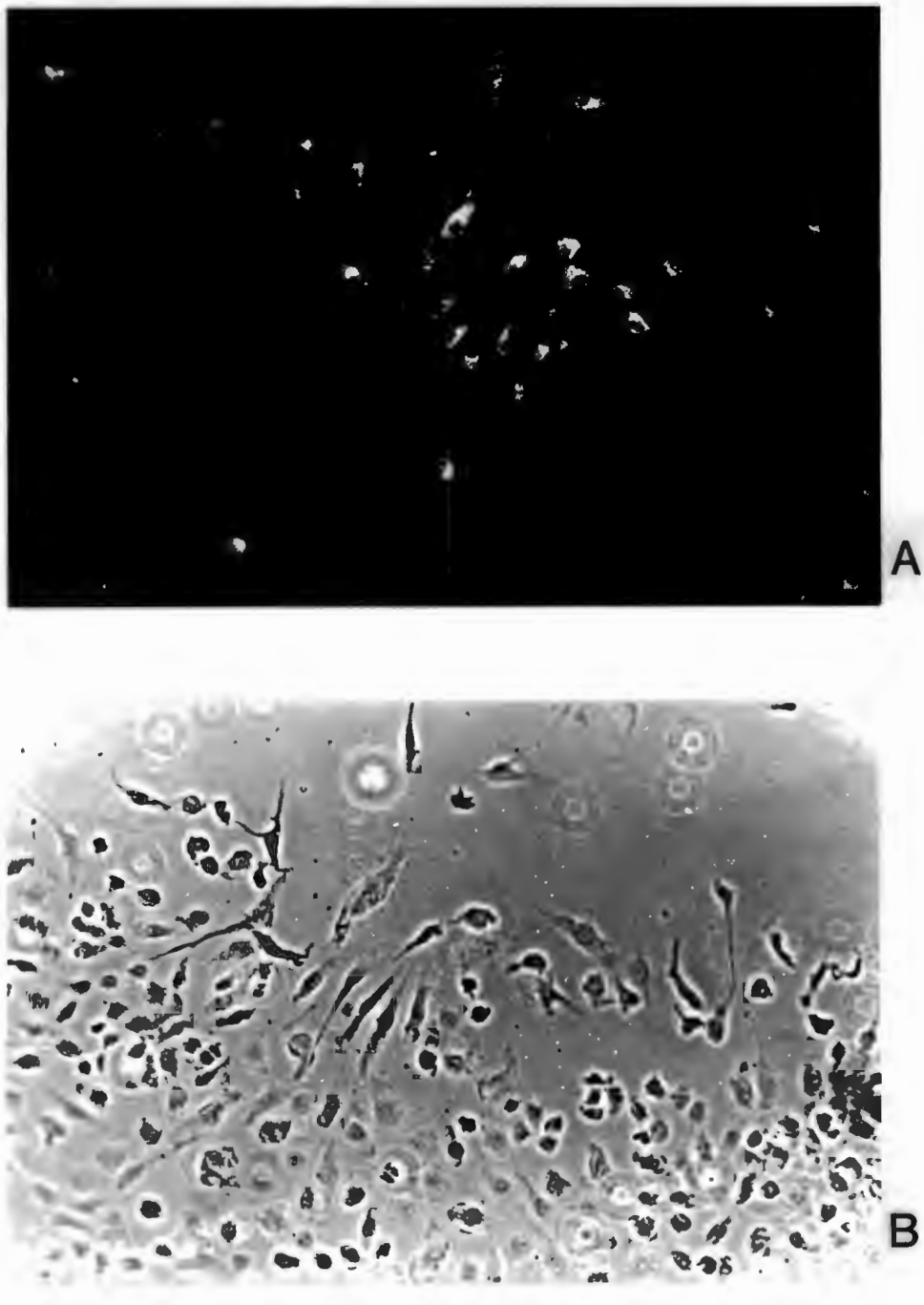


PLATE 3.25: Regeneration in wounded postconfluent E8 monolayers incubated with DiI-LDL (20µg/ml) at 37°C for 4 h. Comparison of the direct fluorescence **A**: micrograph with the **B**: phase contrast image of the same field, reveals that migrating cells entering the denuded wound area have taken up DiI-LDL while the undisturbed postconfluent cells in the "hinterland" have not. Magnifications A, B = 140x.

Plate 3.26 A.B

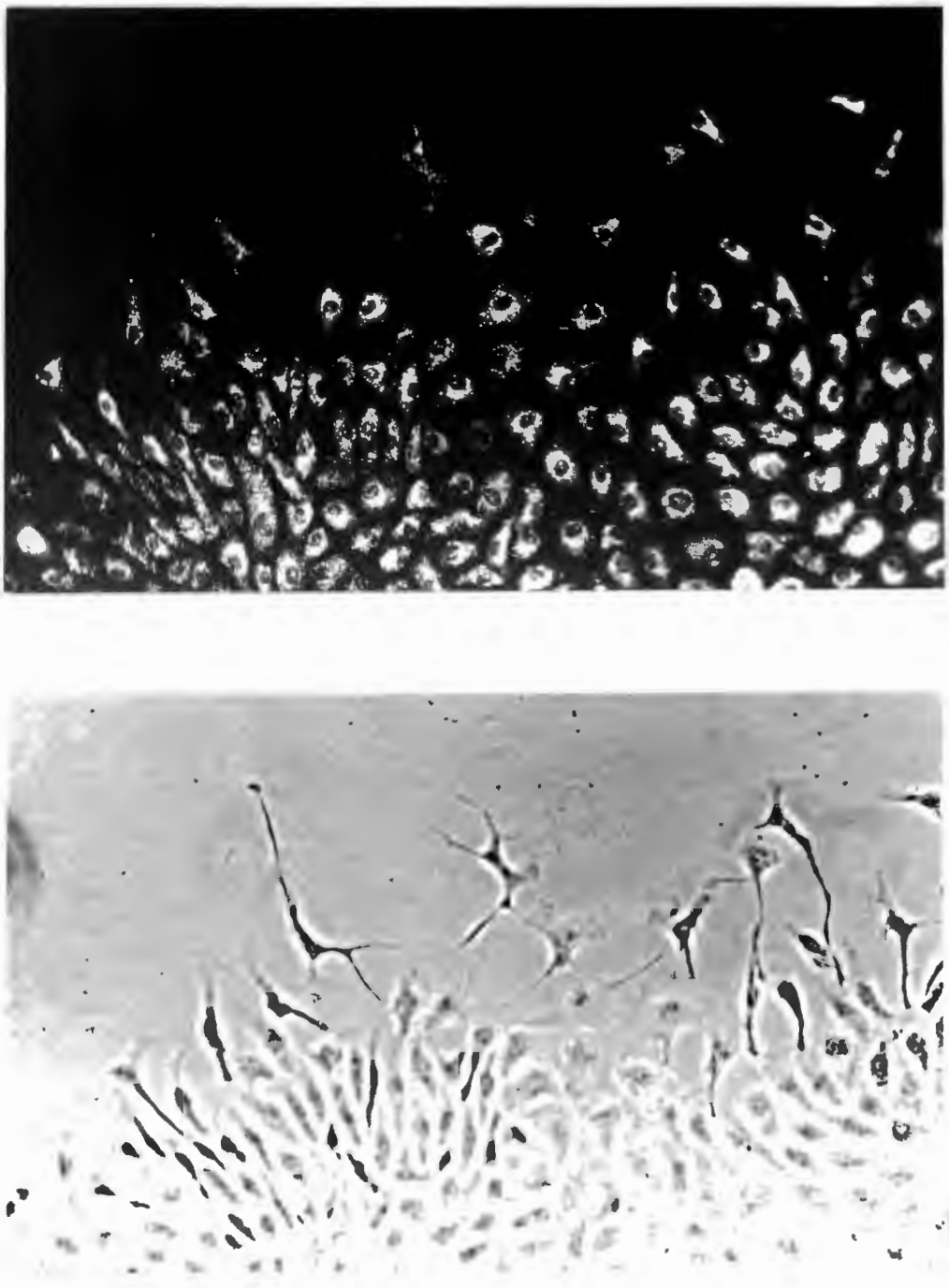


PLATE 3.26: Regeneration in wounded postconfluent E8 monolayers incubated with DiI-AcLDL (20µg/ml) at 37°C for 4 h. Comparison of the direct fluorescence **A**: micrograph with the **B**: phase contrast image of the same field, reveals that both migrating cells entering the denuded wound area and undisturbed postconfluent cells have taken up DiI-AcLDL. Magnifications A, B = 160x.

Plate 3.27 A, B

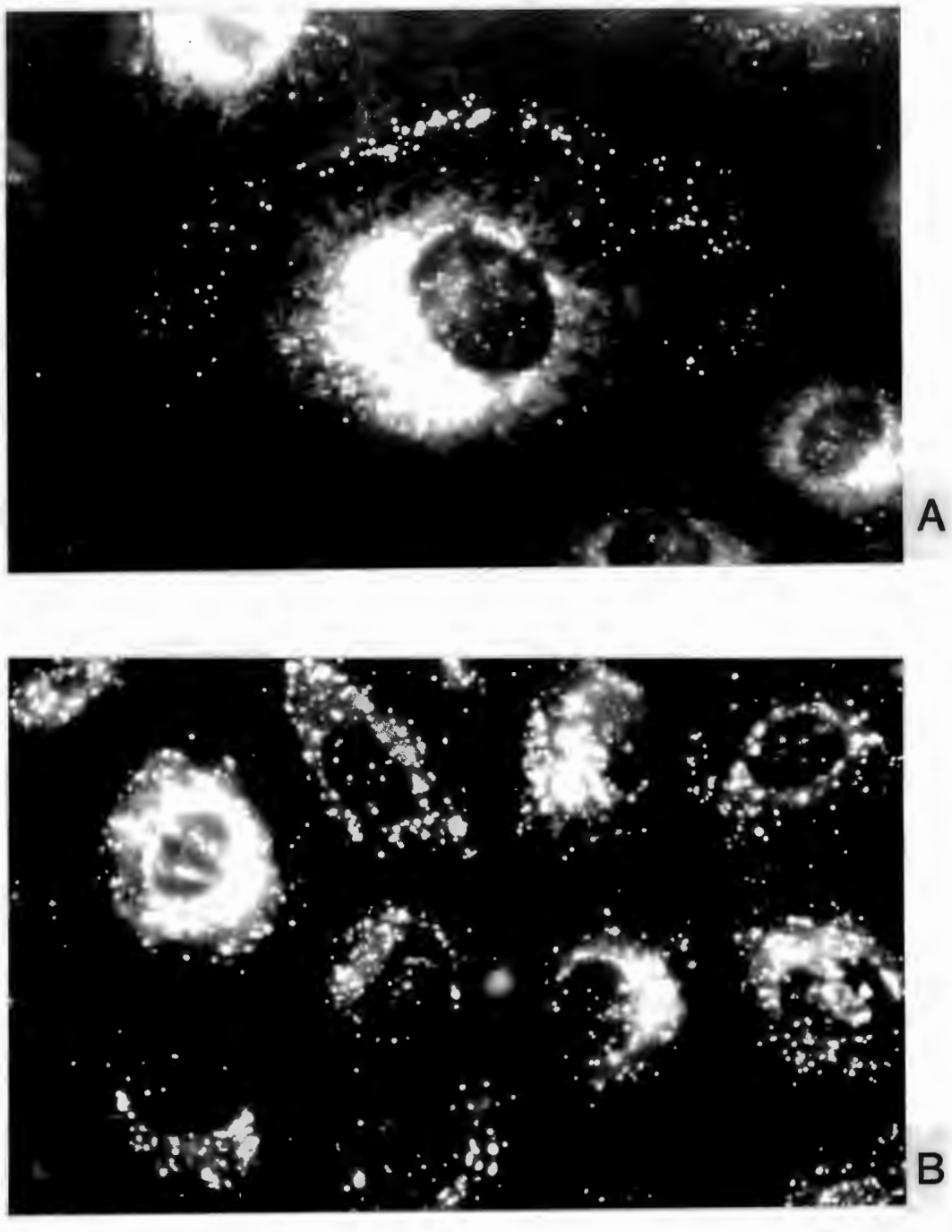


PLATE 3.27: Postconfluent E8 cells incubated with DiI-AcLDL (20μg/ml) at 37°C for 4 h. **A**: E8 cell showing the perinuclear accumulation of DiI label separated from the peripheral large fluorescent foci representing endosomes filled with DiI-AcLDL. The massive perinuclear fluorescence was probably an accumulation of large vesicles as shown more clearly in a number of cells **(B)**: from a postconfluent culture. Magnifications A = 640x, B = 1008x.

DISCUSSION

The existence of distinct, high-affinity, specific receptors to LDL[210] and AcLDL[265] on ECs has been established. Strumpfer[271] has extensively characterized both of these receptors in cultured bovine aortic ECs. Levels of bound and intracellular LDL and AcLDL, and their degradation products in cells showed biphasic response curves to increasing extracellular concentrations of these ligands. The initial phase reflected exponential increases in all the interaction parameters consistent with the saturation of high-affinity binding sites. The second phase was apparent (after saturation) as linear increases in parameters: this represented low-affinity non-saturable binding, which co-existed with the high-affinity component. Competition studies revealed that the binding sites for LDL or AcLDL were distinct. LDL and AcLDL were degraded lysosomally because chloroquine treatment depressed degradation by more than 97%. The equilibrium dissociation constants (Kd) for LDL and AcLDL in E3 were calculated as 20µg/ml and 2µg/ml respectively .

Preincubation of subconfluent ECs with lipoprotein-deficient media served to upregulate the LDL binding sites. E3 cultures exposed to LPDS-MEM for 48 hours showed a three-fold up-regulation of LDL binding sites compared to cells exposed to foetal calf serum-containing media (FCS-MEM). Conversely LDL binding sites in up-regulated cells could be down-regulated by exposure to LDL. AcLDL binding sites, under similar conditions, were not susceptible to regulation.

Since LDL and AcLDL binding sites in ECs were saturable and ligand-specific and, in the case of LDL, also capable of regulation, they could justifiably be considered true receptors.

The extent of binding and metabolism of LDL depended on whether the ECs were subconfluent and actively-growing, as opposed to confluent and quiescent. Relatively high levels of LDL bound to subconfluent cultures, but levels in postconfluent cultures were so low as to be at the limit of sensitivity of the assay. Rates of uptake and degradation modulated with similar kinetics to the binding levels. This point is brought out more clearly by comparing the internalisation indices which describe the efficiency of ligand uptake by receptormediated endocytosis[59] (see RESULTS, p.79). Internalisation indices for both LDL and AcLDL were similar for subconfluent (not sparse) and confluent ECs. This situation contrasts with that found by Coetzee and Chait[59] in macrophages where the internalization index was reported to be several times greater for AcLDL than for LDL. The implication in ECs is that the efficiency of internalisation is relatively constant throughout the maturation of a culture. The possibility of affinity changes between receptor and ligand as a function of morphological state was considered by Strumpfer[271] who found the affinity of the LDL receptor to be similar in subconfluent and postconfluent ECs. Therefore the only major variable remaining which could be responsible for the modulations was the number of receptors. This suggests that both ligands were being endocytosed by the normal route, via coated pits; and that the major regulating mechanism was receptor number and not a decrease of efficiency of internalisation of bound ligand in the case of LDL.

The direct comparison of the binding levels in postconfluent and sparse EC cultures would appear to be justified within the constraints of the assay. Owing to the limits of the radiochemical binding assay, levels of LDL bound to postconfluent ECs may be artificially high. On the other hand, levels on sparse cultures are also probably too high on account of saturable and variable binding to the plastic dishes. A relatively large area of plastic would have been exposed to ligands in sparse EC incubations considering the separation of individual cells. Since both probable errors have the same direction, their effect on the analysis of relative endocytic events is possibly minimised.

Even lower binding of LDL to postconfluent cultures would increase the efficiency of internalisation (and the internalisation index) which runs counter to the suggestions of

Vlodavsky et al.[286] It is interesting to note that Vlodavsky et al. only measured a 2 fold reduction in binding on postconfluent compared to subconfluent ECs (upregulated) using radio-iodinated LDL at 15µg/ml (27.1 and 66.4 ng LDL/mg cell protein). Their LDL binding level for postconfluent cells was very similar to that found in the present study: they both approach the limit of sensitivity of the assay (approximately 20 ng LDL/mg cell protein at 37°C)

Confluent ECs, like fibroblasts,[52, 160, 150] have reduced receptor-mediated LDL binding and metabolism than dividing cultures: but the reduction is more dramatic in ECs. Kenagy et al.[151] reported progressive modulation of receptor-mediated binding and metabolism of LDL in bovine ECs as a function of cell density. Contrary to a previous report by Vlodavsky et al.,[286] they found parallel (i.e. proportionate) decreases in these parameters with increasing cell density.

That the decrease in high-affinity metabolism of LDL in postconfluent ECs was due to markedly reduced receptor numbers on the cell surfaces is supported by the finding that surface-bound LDL was not detected on postconfluent ECs by direct fluorescence microscopy and indirect immunofluorescence microscopy. This conclusion contrasts with the findings of Vlodavsky et al.[77, 102, 286] who argued that the low uptake of LDL in postconfluent ECs is the result of inhibited internalisation, not of binding. They suggested a mechanism to account for this inhibition which invoked structural changes in the surface of postconfluent ECs as opposed to a dynamic regulatory mechanism. It was suggested[283] that the LDL receptor in actively-dividing ECs was free to diffuse laterally, unlike the situation in fibroblasts, and that this lateral diffusion led to endocytosis. This situation was similar for the EGF receptor.[242] In postconfluent ECs, however, lateral diffusion of receptors appeared to be restricted.[284] Vlodavsky et al.[284] argued that altered properties of the cell surface may be responsible for this restriction. The appearance of a specific protein , CSP-60, only expressed on the apical surfaces of postconfluent cells, and the disappearance of fibronectin from these surfaces,[285] provided an indirect structural basis for reduced high-affinity endocytosis of LDL in contact-inhibited monolayers.[283] LDL receptors, albeit reduced in number, were thought to exist on the cell surfaces, but not to mediate ligand internalization in postconfluent cells.

Receptor-mediated uptake of AcLDL occurs in ECs[265] via high-affinity receptors clustered in coated pits.[128] This process actually maximises as ECs reach confluent density as shown by Strumpfer[271] and confirmed in the present study. Receptor-mediated endocytosis in general is likely to take place via coated pits[101] as there is evidence that different receptors co-localise in the same pits.[282, 50, 185] And therefore, since receptor-mediated endocytosis of ligands other than AcLDL must occur in contact-inhibited ECs in vivo, morphological restraints on receptor-mediated endocytosis seem unlikely. These facts are incompatible with the notion of a generalised restriction of lateral mobility of LDL receptors in postconfluent ECs .

In contrast to the findings of Vlodavsky et al.[284] temperature-dependent clustering of LDL receptors in subconfluent ECs was not detected by fluorescence microscopy. Punctate foci, similar in size and pattern to those reported by Anderson et al.[8] to be present on normal human skin fibroblasts, were observed on ECs. Although not as obviously, these foci in ECs were also seen arranged into linear arrays where the cells were sufficiently spread (PLATE 3.10, p.92).

Ligand-Induced Clustering of Receptors

The methodology used in this thesis involved the binding of lipoprotein ligands to unfixed receptors at low temperature. While this had the advantage of ensuring maximum binding, the question of ligand-induced aggregation of receptors at low temperature arose. Anderson et al.[6] performed definitive studies on pre-fixation liganding of fibroblasts with ferritin-labelled LDL. They found no difference in the binding patterns of the ligand on the surface of fibroblasts incubated with F-LDL either before or after fixation. Using fluorescence video intensification microscopy, Maxfield et al.[185] showed that insulin, EGF and α 2-macroglobulin bound

diffusely to fibroblasts at 4°C and that the receptors remained diffuse as long as the temperature was kept at 4°C. At the ultrastructural level, these findings were confirmed for EGF receptors on A431 cells.[136] EGF receptors liganded before fixation were monodisperse at 4°C, and clustered into coated pits at 37°C. EGF receptors are known to undergo ligand-induced clustering at 37°C[62] while most LDL receptors are preclustered at both temperatures in the absence of ligand.[97]

Information more specific for LDL receptors was gained by Barak and Webb[15] using DiI-LDL to measure diffusion coefficients by fluorescence photobleaching recovery (FPR) on fibroblasts at various temperatures. They found that on GM 3348 and GM 2408A at 10 °C less than 20% of receptors diffused and their diffusion rate was extremely slow: only twice that of the 80% considered immobile ($0.5 - 2.0 \times 10^{-11}$ and 0.3×10^{-11} $cm^2 s^{-1}$ respectively). By comparison the diffusion rate at 28°C for the diffusible fraction was 4.5×10^{-11} $cm^2 s^{-1}$, approximately ten times that at 10°C. Extrapolating these rates to 4°C would probably extend the differences sufficiently so the diffusible fraction would have a lower diffusion rate than those receptors considered immobile at 10°C.

Recent work of Tank et al.[272] on the diffusion rates of unliganded LDL receptor in fibroblasts measured by electric field-induced redistribution followed by postfield relaxation essentially support the work of Barak and Webb on FPR of liganded LDL receptors. While not bearing on ligand-induced clustering, they confirm a 10-fold difference in diffusion rates of LDL receptors at 8°C and 37°C. In the present study, unlike that of Vlodavsky et al.,[284] LDL bound to postconfluent ECs at 37°C was not detected by fluorescence microscopical techniques. There was thus no evidence of temperature-induced aggregation of LDL receptors on postconfluent ECs.

The fluorescence studies reported here included repetitions of the techniques of Vlodavsky et al.[284] and Anderson et al.[8] using both fibroblasts and ECs. The latter indirect immunofluorescence procedure resulted in more brilliant ligand-specific fluorescence even though the antibody incubations followed fixation with formaldehyde. These findings were corroborated by direct fluorescence microscopy using DiI-labelled LDL (except in the binding experiments conducted at 37°C in which DiI-LDL was unsuitable for surface receptor probing).

The topographical demonstration of the distribution of AcLDL receptors in postconfluent bovine EC cultures is reported here. The direct fluorescence detection of DiI-labelled AcLDL (DiI-AcLDL), at the light microscope level, was corroborated by indirect immunofluorescence microscopy. The binding sites were randomly distributed, intensely fluorescent punctate foci completely covering the apical surfaces of the cells in the monolayer.

LDL Receptor Regulation in Endothelial Cells

Cholesterol synthesis in actively-growing bovine ECs can be regulated via high-affinity surface receptors for LDL[77] as in fibroblasts.[95] Cholesterol synthesis rates in postconfluent ECs, although much lower than in subconfluent ECs, can be down-regulated further by increased intracellular cholesterol. Although virtually no cholesterol can enter postconfluent ECs via the LDL pathway, it can be taken up in the form of negatively-charged LDL such as acetylated LDL.[77] The uptake of cholesterol via AcLDL or 25-hydroxy-cholesterol which crosses the cell membrane has the effect of depressing the de novo synthesis of cholesterol by inhibiting the activity of HMG-CoA reductase even further.[77] Clearly then, the regulation of cholesterol synthesis by HMG CoA reductase activity is still operational in postconfluent ECs. The activity of this enzyme, the number of LDL receptors synthesized and the activity of ACAT (acyl-Coenzyme A:cholesterol acyltransferase) constitute the classic controls of cholesterol homeostasis in cells.[36]

Kenagy et al.[151] have discussed an interesting phenomenon which occurs in postconfluent bovine aortic ECs; that of an attentuated upregulation of the receptor-mediated degradation of LDL after pre-incubation with media containing lipoprotein deficient serum. This procedure usually results in the reverse transport or loss of cholesterol from cultured cells and leads to a feedback upregulation of receptor-mediated degradation of LDL. They speculate that attenuation of the upregulation response may occur either because reverse cholesterol transport is less efficient in the presence of many cells (i.e. acceptors get saturated) or because there is some intrinsic property of the plasma membrane of postconfluent ECs which limits cholesterol egress. Even though attentuated, the increased LDL receptor-mediated degradation depended on increased internalisation of receptors. This shows that upon demand, postconfluent ECs can respond to cholesterol shortage, like subconfluent ECs, by upregulating the LDL receptors.

Contact-Inhibition and LDL Uptake

The degree of contact-inhibition of growth and associated processes, like LDL uptake, depends upon many factors in cultured ECs. Variant ECs can arise;[170, 171] the substratum upon which cultures are growing can exert an effect;[108] and so can the medium.[171] Bovine aortic ECs have been reported to produce excellent contact-inhibited monolayers[286] and this has been the experience with the E3 and E8 ECs used in the present study as monitored by thymidine indices, LDL uptake and morphology. Much smaller reductions in LDL uptake in postconfluent human ECs (5-fold) were observed by Van Hinsbergh et al.[279] than by Vlodavsky et al.[286] (10-fold) in FGF-dependent bovine aortic ECs. The reduction of uptake (intracellular level plus amount degraded per unit time) of LDL in FGF-independent, postconfluent bovine ECs in the present study was (at best) 30-fold while binding reductions were on average 10-fold. (see Table 3.1) The question therefore arises: were the human cells used by Van Hinsbergh et al. properly contact-inhibited? Thymidine indices reflecting DNA synthetic rates were not given.[279]

Wounding Experiments

Mechanical wounding of the endothelium in vivo,[245, 247] in organ culture[202] and in vitro[300, 94, 284, 67, 139, 252, 250] has been tenaciously studied for many decades since the pioneering work of Poole et al.[209, 208] The interest in endothelial wounding, apart from cell biological mechanisms of cell regrowth, has centred around the importance of endothelial injury in atherogenesis.[224, 225] After simple scratch wounding in vivo[247] or in vitro,[202] ECs migrate rapidly into the wound area and cover the previously denuded area of substratum. Mitosis only starts 24 hours after re-endothelialisation of a narrow scratch wound.[202]

The uptake of native and acetylated LDL by postconfluent and dividing ECs, used as models of normal and regenerating endothelium, has been described morphologically here. While resisting binding and uptake of LDL, postconfluent cultures became heavily loaded with acetylated LDL. Dividing or otherwise active cultures, bound and took up LDL readily while remaining relatively resistant to acetylated LDL. It is tempting to speculate that, should a modified-LDL like AcLDL be found to exist in vivo, confluent ECs would take it up readily and, not being able to store it, pass it on to other cells, like macrophages. This process might be atherogenic given the following assumptions:

1. That modified-LDL exist in vivo

2. That genetic or diet-induced hypercholesterolaemia could lead to the production of localised high concentrations of modified-LDL

Largely as a function of their rapid uptake of modified LDL, macrophages may play an important role in atherogenesis.[182] The high-affinity LDL receptor as well as the high-affinity receptor for modified (or negatively-charged) LDL are present on human monocyte-derived macrophages[276] and mouse peritoneal macrophages.[132] Macrophages degrade modified-LDL up to ten times more rapidly than native LDL, mostly via the AcLDL receptor,[200] and become filled with cholesteryl ester-containing vesicles.[182] Unlike the LDL receptor, the receptor for AcLDL in macrophages is not subject to regulation by cellular cholesterol levels.[241] So uptake of modified LDL may lead to overloading with cholesteryl esters with consequent conversion of the macrophages into foam cells.[182] Cell culture experiments[205] and animal models for atherosclerosis[75, 74] leave little doubt that some foam cells in plaque are of macrophage origin, particularly in the early stages of atherogenesis.

LDL may be modified by covalent linkage of acetyl, acetoacetyl, succinyl, maleyl, and malondialdehyde groups to the exposed lysine residues of apo-B on the surface of the particles.[33] Dextran sulphate and LDL form complexes which bind to the modified-LDL receptor.[182] One effect of these modifications is to produce particles carrying a greater net negative charge than the native LDL. All these modified-LDL may be taken up via high-affinity receptors that are distinct from the LDL receptors. There is some recent ultrastructural evidence that the MDA-LDL and AcLDL receptors on mouse peritoneal macrophages are distinct.[218]

Does something like modified LDL occur naturally, in vivo? Fogelman et al.[80] achieved malondialdehyde modification of LDL in vitro in the presence of aggregating platelets. The MDA-LDL so produced led to a dramatic increase in cholesteryl ester content when taken up by human monocyte-derived macrophages. They speculated that in vivo modification of LDL might occur by reaction with malondialdehyde produced by platelet aggregation or lipid peroxidations. Malondialdehyde is produced by the metabolism of arachidonic acid which accompanies the aggregation and release reactions in platelets. The prostaglandin endoperoxides PGG_2 and PGH_2 are intermediates in the conversion of arachidonic acid to the thromboxanes, which are potent inducers of platelet aggregation. PGG_2 and PGH_2 can also be converted to a C_{17} hydroxy fatty acid (HHT) plus malondialdehyde.[120] Malondialdehyde may also be formed from the breakdown of the arachidonic acid cleaved from phospholipids by phospholipase action.[80]

Although there is still no direct precedent for LDL modification in vivo, highly efficient receptors for the clearance of injected, artificially modified-LDL have been demonstrated in sinusoidal capillary ECs of the rat liver (EC-LDL)[195, 196] and dog liver in vivo (AcAcLDL).[182] Morphological localization of injected, labelled modified-LDL to sinusoidal capillary ECs of liver, spleen and bone marrow of dog, rat and guinea pig has been achieved at the light and electron microscopic levels.[203] Clearance of artificially injected AcAcLDL by the liver of dogs was so efficient that putative, naturally-occuring modified-LDL might be difficult to detect owing to their short residence time in the circulation.[182]

An interesting finding of Henriksen et al.[132] was the modification of human LDL by incubation with rabbit aortic ECs in culture for 24 hours. Nagelkerke et al.[196] discovered that HUVEC cultures can also modify human LDL which elicits a similar modified biological response, viz. extremely swift clearance via the AcLDL receptor on liver sinusoidal capillary ECs. Heinecke et al.[130] reported transition metal catalyzed modification of LDL by human arterial smooth muscle cells in culture. Steinberg and co-workers[268] have suggested a mechanism for the production of such EC-modified-LDL (EC-LDL) which depended on the presence of traces of transition metal cations in the medium, as was later discovered. EC-LDL behaved similarly to AcLDL, being taken up and degraded via the same receptors on macrophages[132, 200] and ECs.[196] EC-LDL elicited biological responses different from LDL:

1. Macrophages degraded EC-LDL 3-10 times more rapidly than LDL[200, 132] via the AcLDL receptor.

2. EC-LDL was degraded by the LDL receptor, but less rapidly than LDL.[200]

Physical modifications included a higher hydrated density (from 1.036 to 1.070 g/ml) and a higher negative charge (determined electrophoretically) than those of native LDL. The chemical modifications which underlie these changes include phospholipid peroxidation by free radicals catalysed by transition metal cations (Fe^{2+}, Cu^{2+} and Zn^{2+}). This peroxidative cleavage of fatty acids is non-specific and does not cleave entire fatty acyl chains. In addition to, and separately from this peroxidative cleavage, phosphatidylcholine is hydrolysed to lysophosphatidylcholine by phospholipase A_2. The enzymic activity probably resides in the LDL particle itself, since the hydrolysis occurs in the absence of cells or cell associated fluids.

These changes to the LDL particle may be stopped by any one of the following agents: free radical scavengers (antioxidants like Vitamin E and butylated hydroxytoluene), chelators like EDTA in sufficiently low concentration to remove transition metals without totally depleting calcium, and parabromophenacyl bromide (pBPB) which selectively and totally inhibits phospholipase A_2. Of these processes, the metal catalyzed peroxidation is mandatory for modified LDL production. If the concentration of transition metal cations is sufficiently high and reaction time prolonged, metal catalysis alone will modify LDL. The other processes are essential in vitro as enablers of the metal peroxidation but cannot, on their own, modify LDL, since, in the absence of metals, the process will not take place.

Apo B itself is modified by peroxidation: its lysine residues are either lost, modified or both. And since the LDL receptor has active sites for domains on the Apo B molecule,[157] this explains why the EC-LDL binds to the LDL receptor with a much lower affinity than LDL. It also accounts for the existence of a distinct receptor (or receptors) for modified LDL.[200]

Negatively-charged, particulate LDL, termed A-LP, have been isolated from human atherosclerotic plaque by Hoff and co-workers.[135] This constitutes indirect evidence for the modification of LDL in vivo amd raises the intriguing possibility that the process may be taking place cryptically in micro-environments, e.g. within the intima.

If they existed in vivo, what relevance could modified-LDL have to the pathophysiology of atherogenesis? If MDA-LDL forms in vivo by reaction with malondialdehyde produced by platelet aggregation at the site of vascular injury, this may be atherogenic. A speculative scenario, whereby LDL modified by macrophages, smooth muscle cells[130] or ECs could be atherogenic was suggested:[33] LDL accumulations in the intima following overload, are peroxidized and taken up by macrophages which eventually become foam cells filled with cholesteryl oleate droplets. Foam cells filled with cholesterol oleate are a source of the yellow grumous lipid which characterises early atheromatous lesions.[95] Indirect evidence comes from the finding of Pitas et al.[205] that foam cells from the medial layer of atherosclerotic rabbit aorta have receptors for acetoacetylated LDL (AcAcLDL) as well as βVLDL. Since macrophages have both these receptors, this finding suggests that macrophages may be precursors of foam cells. SMC from the same aortae did not have receptors for βVLDL.

CHAPTER FOUR

LDL Receptors on Active and Contact-Inhibited Aortic Endothelial Cells, and on Fibroblasts: Ultrastructural Analysis with Colloidal Gold Probes

INTRODUCTION

In the previous chapter, the high-affinity binding of LDL and AcLDL to the surfaces of subconfluent and confluent ECs was analysed by fluorescence microscopy. LDL was seen bound to the surface of subconfluent ECs in small punctate foci, but it was not detected on the surface of postconfluent monolayers. These findings were corroborated by radiochemical assays of LDL binding and metabolism by cells in both morphological states.

This chapter addresses itself to the following questions:

1) Can LDL be detected on postconfluent monolayers at the ultrastructural level? The fluorescence techniques applied (see Chapter 3) may not have been sensitive enough to detect a low but specific binding level.

2) What is the nature of the fine focal binding of LDL to subconfluent ECs? Are LDL receptors (LDL-Rs) clustered or dispersed, or both?

3) Do the foci correspond with topographical ultrastructural features?

The methodological approach to the ultrastructural analysis of lipoprotein interactions with cell surfaces was a crucial consideration in this work. Native lipoproteins are not sufficiently electron-dense to generate sufficient contrast in the TEM for easy visibility. Therefore methods of staining them or coupling them to electron-dense markers (or tags) have been developed by others. Anderson et al.[6] adapted the methods of Kishida et al.[153] to produce ferritin-conjugated LDL (F-LDL) which was used as a probe in one of the earliest ultrastructural studies of the LDL receptor (LDL-R). The conjugation was a lengthy process involving chromatographic purification of monomeric ferritin, subsequent activation of the ferritin with pure glutaraldehyde, followed by conjugation with LDL and repurification of the probe by ultracentrifugation. ^{125}I-labelled LDL, detected by electron microscopic autoradiography (EM-ARG) is a second approach that has been used to localise LDL in fibroblasts.[49] However the limited resolution of EM-ARG (approximately 60nm[239]) and exposure times of weeks have prompted the search for more rapid, higher resolution techniques. In transcytosis studies of rat vessels, LDL has been visualised by direct staining with diaminobenzidine and tannic acid, and by immunoperoxidase techniques.[280]

All of the above means of rendering LDL visible at the ultrastructural level were applied to thin-sectioned material. Internalised F-LDL tended to disappear against the background in stained sections, while in unstained sections it was difficult to identify the intracellular sites of F-LDL binding. With the exception of autoradiography, none of the above methods was especially suited to topographical studies of LDL binding.

Colloidal Gold Marking

The advent of colloidal gold-labelled macromolecular probes has revolutionised ligand-receptor studies by electron microscopy.[68] Routine technology for the production of gold colloids of controlled and uniform size has been evolved[83] and refined.[260] A wide variety of macromolecules, including antibodies,[76] growth factors,[121] avidin and staphylococcal protein A,[275] can be conjugated to colloidal gold particles by simple methods.[reviewed 228] Colloidal gold probes are ideal for topographical and intracellular studies and permit resolution better than 5 nm.[260]

Colloidal gold-low density lipoprotein conjugation was perfected by Handley et al.[122, 123] who used the conjugates as probes for LDL-Rs (LDL-Rs) in human skin fibroblasts[122] and later in hepatocytes.[123] The conjugate, Au-LDL, unlike F-LDL, is an intensely electron-dense probe

owing to the high atomic number and consequent electron scattering power of gold. Colloidal gold probes stand out very clearly when viewed in the TEM even when surrounded by other relatively electron-dense ultrastructure stained with heavy metals like osmium, uranium and lead. Au-LDL consists of single gold particles approximately 20 nm in diameter surrounded by 7-9 LDL particles bound electrostatically during a simple, short incubation involving no harsh chemical treatment. The probe preparation is freed of unbound LDL and concentrated for use by simple centrifugation. The probe is stable at high salt concentrations (0.5 M NaCl) and over a wide pH range (4 - 9), but it tends to aggregate and cannot be stored for more than 24 hours.

Au-LDL binds specifically to the LDL-R as established by competitive inhibition of binding by excess unlabelled LDL, and by absence of binding in FH homozygote fibroblasts which lack LDL functional receptors.[122] If Au-LDL is used at low concentrations, binding to high-affinity receptors is favoured. The overall diameter of the probe is approximately 60 nm, three times the diameter and 27 times the volume of native LDL. It is too large to enter caveolae which have an average neck diameter of 50 nm[84] and so it cannot be used to trace endothelial vesicular routes of receptor-independent endocytosis or transcytosis.

Probing the LDL Receptor

Analysis of the binding of F-LDL to the surface of fibroblasts by thin sectioning was done by Anderson et al.[6] It was a laborious means of gathering information from a sample of surface necessarily limited by the nature of the technique. Faster and more accurate topographical methods using plan views of the cell surface have superseded thin section methods. One approach to the topographical analysis of Au-LDL interaction with cell cultures was that of Robenek and co-workers.[215, 217] They used the platinum/carbon replica technique to demonstrate lipoprotein interactions with receptors on human skin fibroblasts and macrophages.[215, 133, 218, 243] The technique exploited the ability of replicas to embed or trap the colloidal gold labels of probes bound to the cell surface. After the replica had been cast, the cellular material was digested away leaving behind the gold particles embedded in the replica in their original positions with respect to topographical ultrastructure. The preservation of fine surface detail in replicas was sufficiently good to allow the identification of receptor-bound Au-LDL within coated pits. Strictly, the position of gold on the cell surface gave positional information about the ligand which may or may not have been bound to a receptor. Since non-specific binding or even pre-binding aggregation of LDL may occur, a means of checking the receptor positions by some other means might confirm the positional information gained by ligand probing. It is unlikely that a second method would duplicate artefacts, so similar binding results from two different methods would considerably strengthen confidence in the results considerably.

Replicas give only topographical information about cells and so take limited advantage of plan projections in the TEM. Variations in the thickness of the cell cannot be analysed by replica techniques and internal detail cannot be detected directly. The use of whole-mounted cells, labelled with gold-conjugated probe molecules has been pioneered by Hopkins and co-workers.[137, 138, 136, 275] Mapping of transferrin and EGF receptors during endocytosis has been elegantly executed in such systems. Hopkins has developed shadowing techniques which allow discrimination of surface-bound and intracellular gold probes, so that replication for selective viewing of surface-bound probes is unnecessary.

Whole-Mounts

Dried cell cultures have been viewed whole by high voltage transmission electron microscopy (HVEM) by others.[41] The high voltage electron beam readily penetrated the relatively thick nuclear and perinuclear regions of cells. Clear images of organelles such as mitochondria,

endoplasmic reticulum, polyribosomes, microtubules, and coated vesicles were easily obtained.[41] The whole-mount approach avoids the stereological limitations of thin sections. To reconstruct a three-dimensional image of a cell from the two-dimensional information in sections, many serial sections must be analysed and the information then synthesized to produce the third dimension. Clearly, the resolution of surface details is a function of the number and thickness of the sections. This imaging process is tedious and of relatively low resolution. Using whole-mounted cells, stereo pairs of images may be recorded which allow rapid, high-resolution and three-dimensional analysis of surface and internal information.[41]

Although the HVEM yields the best results, the conventional TEM has been used at accelerating voltages of 80-100kV for the study of whole cells.[42, 40] The thinner, peripheral cytoplasmic regions of cultured cells may be satisfactorily imaged. Whole-mounted ECs cultured on coated grids were viewed by Ausprunk[12] at 80-100kV when she described the stages of cell spreading.

Whole-mount preparations of cultured porcine ovarian granulosa cells[137, 275] and human epidermoid carcinoma A431 cells[138, 136] have been used for colloidal gold-based cytochemistry at the electron microscope level. In the latter, gold-labelled monoclonal antibodies to the transferrin receptor were used to map the surface distribution of transferrin receptors at the edges of A431 cells and also to trace the itinerary of receptors during endocytosis. The methodologies of Handley et al.,[122] Robenek et al.[215] and Hopkins[138, 137, 275, 136] were modified and used in the present study to identify and localise the LDL-R on fibroblasts and endothelial cells. The work of Hopkins improved on earlier whole-mount studies by faithful preservation of cell surface morphology. Well-preserved topographical ultrastructure was mandatory for the interpretation at high resolution (better than 5 nm) of interactions of electron-dense probes with the cell surface.

LDL Binding and Endocytosis in Human Skin Fibroblasts

Binding and endocytosis of LDL via high-affinity receptors has been extensively studied in human skin fibroblasts. Two ultrastructural methodologies have been used . Anderson and co-workers[6, 5, 2, 7, 3, 199, 49, 50] and Via et al.[282] have used thin sections for analysis. Topographical EM studies using replicas have been carried out by Robenek and co-workers[217, 219, 215, 216, 133] and by Orci et al.[199] using freeze-fracture techniques. Two different descriptions of the distribution of LDL-Rs on the surfaces of fibroblasts have emerged from these analyses.

The work of Anderson et al. and of Orci et al.[199], mentioned above, indicated that both dispersed and clustered LDL-Rs exist on the surface of the normal active fibroblast. Clustered receptors occur over coated regions of the plasma membrane and lead to internalisation of LDL by receptor-mediated endocytosis (RME) via the coated pits. The dispersed population is ill-defined and poorly understood. It may represent newly-synthesized and/or recycled receptors which have been re-inserted into the plasma membrane and which will migrate into clusters. Receptor-clustering into coated pits, at least for a large population of LDL-Rs on human skin fibroblasts, is independent of ligand binding. In this respect it differs from the behaviour of, for example, the EGF receptor where ligand-induced clustering of receptors does occur and is necessary for endocytosis via coated pits.[62] Ligand-induced clustering of the LDL-R and subsequent endocytosis of the ligand/receptor complexes was examined by Basu et al.[16] and recently discussed by Goldstein et al.[97] as summarised below. Under the influence of the ionophore monensin, fibroblasts could be induced to "trap" 50% of their LDL-Rs in the absence of ligand. This was interpretted as meaning that 50% of the LDL receptors did not require ligand binding for endocytosis via coated pits. The remaining receptors (50%) on the surface of cells in the presence of monensin, were thought not to be located in coated pits but capable of clustering in coated pits and endocytosis upon binding of LDL. Preliminary ultrastructural studies by Goldstein et al.[97] have not confirmed this, showing receptors clustered into coated pits in the presence of monensin and absence of LDL. Nevertheless, the view that some LDL-Rs

(30%) are distributed diffusely on the cell surface outside coated pits persists with these workers. It appears that the reasons why only 50% of LDL-Rs will recycle in the absence of LDL are still unknown.

An alternative view was presented for the fibroblast by Robenek et al.[215] who suggested that in the plasma membrane only pre-clustered receptors exist and that there is no population of randomly-dispersed single receptors. They postulated that, in the sequence of endocytic events, LDL-Rs exist as aggregates or clusters which result in (i.e. cause) the formation of coated membrane regions or pits beneath them.[219]

LDL Binding and Endocytosis in ECs

How the endothelial cell conforms to these models is unknown since little information is available.[189, 119] High-affinity binding and endocytosis of Au-LDL in human venous and arterial endothelial cells in culture was analysed by transmission electron microscopy of ultrathin sections by Mommaas-Kienhuis et al.[189] At 4^{0}C, Au-LDL was bound to the surface in clusters only. Of these clusters approximately 50% were associated with coated pits and approximately 50% with uncoated lengths of plasma membrane. No dispersed Au-LDL was seen bound to membranes. These data from endothelial cells support the notions of Robenek et al.[219], based on fibroblast studies, that recycled LDL-Rs exist in a preclustered form on the cell surface. It is possible that topographical analysis of receptor distribution by thin section techniques may yield incomplete data owing to inherent sampling problems. It is possible that a diffuse population of surface receptors, which does not occur on all cells or on all regions of any one cell, might be poorly represented or even escape detection in thin sections.[215]

No topographical study of LDL-R distribution based on plan views of endothelial cells has been reported yet, so it was decided to undertake one using bovine aortic ECs. Au-LDL was chosen as the most suitable probe to label the surfaces of subconfluent and postconfluent bovine aortic ECs.

The distribution of receptors was mapped first in fibroblasts in order to standardise the techniques. Normal and mutant human skin fibroblasts were labelled with Au-LDL. One of these mutants, GM 2408A, although capable of binding LDL , has an abnormal LDL-R which cannot enter the cell. It was included in this study because the distribution of LDL-Rs on its surface was expected to differ from that on normal fibroblasts as detected by other analytical systems, e.g. fluorescence microscopy and thin section electron microscopy. A receptor-negative mutant, GM 2000, which failed to bind LDL, was also included in the present study as a negative control.

In addition to Au-LDL probing, the receptors were also localised by means of monoclonal antibodies to test for possible probe-induced variations in distribution. Two co-existent populations of receptors were found by both techniques: dispersed and clustered receptors. The Au-LDL probe was thus cleared for use with ECs, where the same dual receptor population was demonstrated in subconfluent ECs. The clustered Au-LDL was shown by ultramicrotomy to be localised over coated pits. Postconfluent ECs bound virtually no Au-LDL.

MATERIALS and METHODS

Cell Cultures

The cell cultures used in this chapter were E8 endothelial cells, normal human skin fibroblast FGo, LDL-receptor negative mutant GM 2000 and LDL-receptor internalisation defective mutant GM 2408A (J.D.). All these cultures have been more fully described in Chapter 2 (pp.35-37). Incubation of cells with media containing lipoprotein-deficient serum (LPDS) served to upregulate LDL-R numbers (see Chapter 3).

Cytochemistry

For the whole-mount cytochemistry conducted here, the cells were cultured in styrene plastic dishes of two kinds. Plain dishes were used for experiments using lipoproteins labelled directly with gold colloid. For immunocytochemical tests, cells were seeded into special dishes which allowed four individual immunocytochemical procedures to be conducted in one dish (for details see **Appendix** to this chapter). This had obvious advantages in reagent economy, particularly with IgG-C7, a monoclonal antibody against the LDL-R.

The IgG-C7 Antireceptor monoclonal Antibody

A monoclonal antibody to the LDL-R was kindly donated by Drs J.L. Goldstein and M.S. Brown (Dallas, Texas, USA). The monoclonal antibody, IgG class 2B, was raised by injecting mice with octylglucoside solubilized LDL receptors from bovine adrenal cortex. Details of its production and purification were given by Beisiegel et al.[17] Lyophilized IgG-C7 (C7) was reconstituted with distilled water and divided up into convenient aliquots which were frozen and stored at -70^{o}C until required for use. A monoclonal antibody, IgG-2001, raised against a viral antigen was used as a control for IgG-C7. IgG-2001 was also a gift from Dr. J.L. Goldstein. It was substituted for C7 in incubations with the three fibroblast cell types used.

Radio-iodinated C7 has been used to probe the LDL-R itinerary during recycling,[35] and native C7 can be detected by immunofluorescence.[17] Although feasible, the use of C7 as a receptor probe at the ultrastructural level has not been reported. Part of the reason for its use in this study was to explore that feasibility.

Immunofluorescence detection of IgG-C7 Antireceptor Antibody

C7 was used to probe for LDL-Rs on the surfaces of normal (FGo) and receptor-negative (GM 2000) human skin fibroblasts to establish normal functioning of the antibody. An immunofluorescence microscopical method was used (144). Cells were incubated with C7 at 10µg /ml in 0.5% BSA in MgPBS for 60 minutes at 4^{o}C. After washing off excess C7, the cells were fixed in 3% paraformaldehyde. C7 was detected with the IgG fraction of rabbit anti-mouse IgG antiserum conjugated with FITC (RAM-FITC) (Bio-Yeda, Rehovot, Israel). Epifluorescence microscopy was performed as detailed in Chapter 2.

Indirect Immunocytochemical Detection of C7 with an Immunogold Probe

Cells were held at 4°C during incubations with C7 and during fixation to prevent receptor movement in the plasma membrane. All solutions used were pre-chilled before use in these steps. A 30 minute incubation in 3% BSA was included to reduce nonspecific binding of the gold-labelled goat anti-mouse IgG antiserum (IgG fraction) conjugated to 15 nm colloidal gold (GAM-G15, Janssen Life Sciences Products, Beerse, Belgium). Cells were incubated for 60 minutes at 20-25°C with GAM-G15 diluted 1:20 with tris-HCl buffer (pH 8.2) containing 1% BSA. After washing to remove unbound gold probes, the cells were fixed in 2.5% glutaraldehyde in MgPBS. Whole-mounts were then prepared for transmission electron microscopy (see **Appendix** to this chapter).

Plate 4.1

PLATE 4.1: Colloidal gold particles mounted on a poly-l-lysine-treated formvar-coated grid. The particles are mostly monodisperse, although occasional pairs and triplets are seen (small arrowheads). Particle diameter is 18.5 nm ± 1.3 nm S.D.
Transmission electron micrograph. Magnification = 60 000x

Plate 4.2

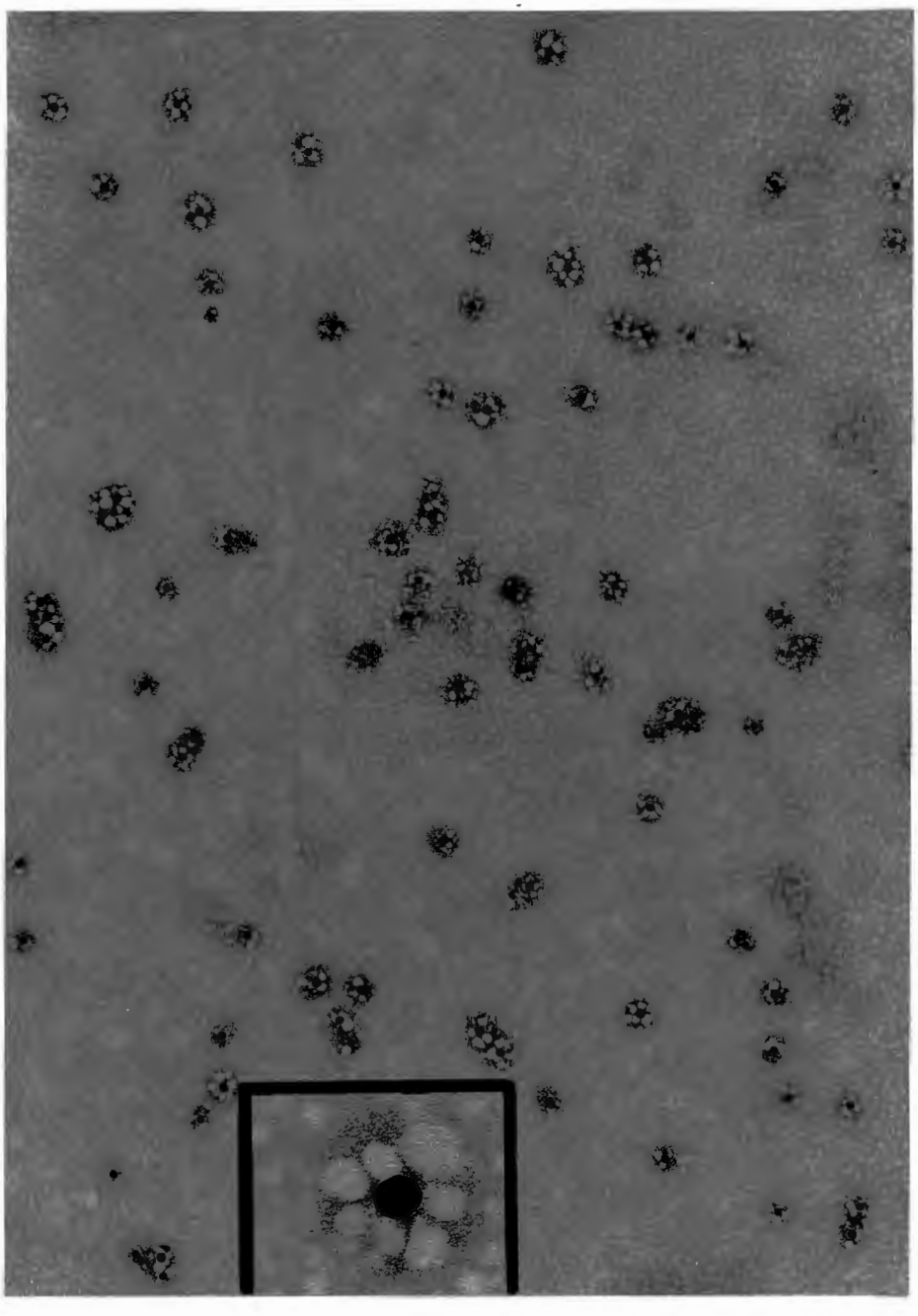


PLATE 4.2 Transmission electron micrographs of Au-LDL probes on poly-l-lysine treated formvar-coated grids. The Au-LDL is mostly monodisperse but occasional pairs and triplets are seen. Free LDL is rare (arrow). The background contains many albumin molecules from the MgPBS diluent which contained 0.2% BSA. Magnification = 60 000x
Inset: Au-LDL probe surrounded by 7 LDL molecules. Magnification = 350 000x

Preparation of gold-labelled LDL (Au-LDL)

The 20 nm diameter colloidal gold particles were prepared according to the method of Frens[83] as modified by De Mey.[68] The colloid was checked for aggregation and size on poly-l-lysine treated formvar-coated grids[68] and found to consist of monodisperse gold particles 18.5 ±1.3nm in diameter (PLATE 4.1). Au-LDL was prepared according to the method of Handley et al.[122] as modified by Robenek. et al.[219] Briefly, 100µg of freshly prepared human LDL (see Chapter 3, p.68) was mixed with 5 ml of colloidal gold suspension. After centrifuging the conjugate against a 35% sucrose cushion in order to concentrate the probes, the yield was centrifuged three times in 0.2% BSA in MgPBS at 10 000xg for approximately 60 minutes at 4°C to remove unconjugated lipoproteins. A red, mobile pool of probes collected on the bottom of the centrifuge tube. This pool, the volume of which was usually less than 1 ml, was diluted to 13 ml in subsequent washes. After the final centrifugation the sediment, containing no more than 100µg of LDL was taken up into a final volume of 1 ml of 0.2% BSA in PBS. Very little free LDL was detected upon examination of the final probe by transmission electron microscopy (PLATE 4.2).

Incubation of Au-LDL with Cultured Cells

Au-LDL was diluted 1:10 with MEM-LPDS for the labelling experiments, so the final concentration of LDL was less than 10µg/ml. Because each gold particle bound between 7 and 9 LDL particles, the concentration of LDL on a particle basis would be 7 - 9 fold lower for Au-LDL than for native LDL at any given protein concentration. The Kd for LDL on the fibroblasts receptor is given as 2µg/ml at 4°C (See Chapter 3, p,69).[99] The concentration of Au-LDL used in these experiments strongly favoured high-affinity binding to LDL-R. Au-LDL would therefore be bound almost exclusively to high-affinity receptors. This last point was strongly borne out by the almost total lack of Au-LDL bound to receptor-negative cells, which are known to take up LDL by receptor-independent means.

Au-LDL diluted in MEM-LPDS (at 4°C) was added to pre-cooled cells and incubated for 60 minutes at 4°C. Cultures were thoroughly washed at 4°C (see chapter 3, Materials and Methods) to remove all unbound probes. Cold 2.5% glutaraldehyde in 0.1M cacodylate buffer, pH 7.4 was used to fix the cells at 4°C for 60 minutes. Fixed cells were immediately processed for whole-mount transmission electron microscopy.

Whole-Mounted Cells for Transmission Electron Microscopy

Details of the methods used for preparing whole-mounted cells for transmission electron microscopy are given in the **Appendix** to this chapter (p. 166).

Embedding and Ultramicrotomy of Au-LDL-treated Cell Cultures

Cells for thin sectioning were incubated with Au-LDL exactly as for whole-mount preparations. After fixation in glutaraldehyde cells were processed for embedding in resin, and for ultramicrotomy as described in Chapter 2.

RESULTS

Fibroblast Standardisation

Normal human skin fibroblasts have been labelled with Au-LDL by Robenek and co-workers[215] and have provided a useful standard against which to test the gold labelling systems used in this study. The mutant human skin fibroblasts GM 2000 (receptor-negative) and GM 2408A (internalisation-defective) were used as controls as follows. GM 2000 served as a negative control since it is known to bind virtually no LDL owing to a lack of functional surface receptors. GM 2408A acted as an alternative positive control as it lacked the ability to internalise bound LDL, and hence a surface distribution of receptors different from normal fibroblasts was expected. Normal fibroblast FGo was used as a positive control for LDL-R studies.

Immunocytochemical methods for localising LDL-R using antireceptor monoclonal antibody, IgG-C7, and direct methods using gold-labelled ligand, Au-LDL, were tested on these three fibroblast cell types. The control monoclonal antibody, IgG-2001, which was not specific for the LDL-R, was substituted for IgG-C7 in experiments with the fibroblasts. In all cases its binding levels were negligible. The immunocytochemical methods using C7 have been used to confirm immunologically that the Au-LDL binding pattern did, in fact, represent the true distribution of receptors. The C7 bound to LDL-R was in turn detected with a gold-labelled goat anti-mouse IgG antibody (GAM-G15) and visualised in the TEM.

a) Immunofluorescence Microscopy of C7 Bound to Normal and Mutant Fibroblasts

C7 bound to the receptors on the surface of FGo cells at $4^{o}C$ was seen by indirect immunofluorescence microscopy (IIFM) as uniformly distributed fine punctate foci which were faint but otherwise similar to the binding pattern of LDL (see PLATE 3.23, Chapter 3), also seen by IIFM. The focal C7-specific fluorescence was easily detectable by eye. Unfortunately, the fluorescence was too faint to be recorded photographically, even on film rated at 1600 ISO and processed for maximum sensitivity. No punctate fluorescence was detected in FGo controls from which C7 was omitted. Receptor-negative cells, GM 2000, had no C7-specific fluorescence. It was thus established that the C7 monoclonal antibody was functioning effectively.

b) Colloidal Gold Immunoelectron Microscopy

On FGo cells, probed with C7 at $4^{o}C$, LDL-Rs were distributed as clusters, loosely or tightly organised and ranging in size from 200 - 500nm in diameter (PLATE 4.3 B). A dispersed population of receptors randomly distributed amongst the clusters was seen (PLATE 4.3 A). Clusters were often roughly ring-shaped with empty central areas. The gold particles represented receptor-bound C7 because receptor-negative GM 2000 cells probed in the same way were almost completely devoid of gold particles (PLATE 4.4 A,B). Receptor expression and consequently C7 binding to the surface of these cells was effectively nil. A dispersed population of receptors was detected on GM 2408A cells probed with C7. Clustered receptors were not seen (PLATE 4.5 A,B). The receptors were uniformly distributed over the surfaces of the cells and groups of more than three gold particles were extremely rare. This distribution resembled the diffuse population seen amongst the clusters on FGo cells. GM 2408A has been shown by other methods[7] (see DISCUSSION, this chapter) to bind hardly any LDL in coated pits. Therefore the dispersed binding of C7 to receptor as seen here (PLATE 4.5 A,B), and later with the gold-labelled LDL, validates the labelling of the disperse population of C7 bound to FGo shown in PLATE 4.3 A. It is thus unlikely to be the result of low-efficiency binding of gold-labelled antibodies to clusters of C7-probed receptors which only appear to be dispersed because many individual receptors have escaped labelling.

c) Au-LDL Cytochemistry : Direct Ligand Probe

Once the the binding pattern of C7 to the three fibroblast cell types had been established, the interactions of Au-LDL with these cells were visualised in the TEM. Fibroblasts were incubated with Au-LDL at $4^{o}C$ and the surface distribution of ligand-receptor complexes analysed. The concentration of Au-LDL was below 10µg/ml (see Materials and Methods of this chapter) in order to favour receptor-mediated binding. The findings confirmed and amplified the information gained by the immunocytochemical localisation of LDL-R with C7 and GAM G15 owing to the much higher efficiency of marking of the receptors by the Au-LDL method.

Normal fibroblasts (FGo) bound Au-LDL as large clusters (200-500 nm in diameter), often ring-shaped and distributed randomly over the cell surfaces (PLATE 4.6A-D). The clusters were sometimes in groups (PLATE 4.6 A) or appeared compound (PLATE 4.6 C,D) as if two or more clusters had partially coalesced. The occurrence of groups of receptors was not uniform from one part of a cell to another, nor from one cell to another. This variability has been reported by Robenek et al.[219, 217] The plasma membrane beneath the clusters appeared smoother than the surrounding areas and seemed slightly depressed. The depression was evident by the reduced electron density of the area and by the fact that individual Au-LDL particles were not haloed by the metal-shadowing as were the more elevated particles. Close examination of the gold probes (PLATE 4.6 D) will reveal that most of them have haloes Most surface-bound probes receive haloes after low-angle metal-shadowing.[275, 136] Those on the rims of clusters have clear haloes while probes in low-lying areas or pits often lack haloes (PLATE 4.6 A). Probes lying in depressed areas may have escaped the low-angle shadowing with platinum/gold.

Monodisperse Au-LDL probes were also observed on parts of the cell surfaces, either apart or interspersed amongst clusters. These monodisperse probes constituted a considerable part of the total probe population (PLATE 4.6 B,C). Aggregates of two, three, or four were seen but were much less common than monodisperse probes. GM 2000 cells bound virtually no Au-LDL (PLATE 4.7 A-C). The non-specific binding level of Au-LDL was thus extremely low and could not explain the monodisperse population seen on both FGo and GM 2408A. Au-LDL bound to GM 2408A receptors mainly as monodisperse probes and with decreasing frequency, as doublets, triplets and groups of four. Groups of more than three particles were rare (PLATE 4.8 A-D). The disperse Au-LDL did not appear to lie in depressed areas, nor was there evidence of loosely-organised groups. The overall impression after examining large numbers of cells was that Au-LDL probes were monodisperse on the surface of GM2408A cells. This result demonstrates the ability of the probe to label non-clustered receptors effectively.

Plate 4.3 A

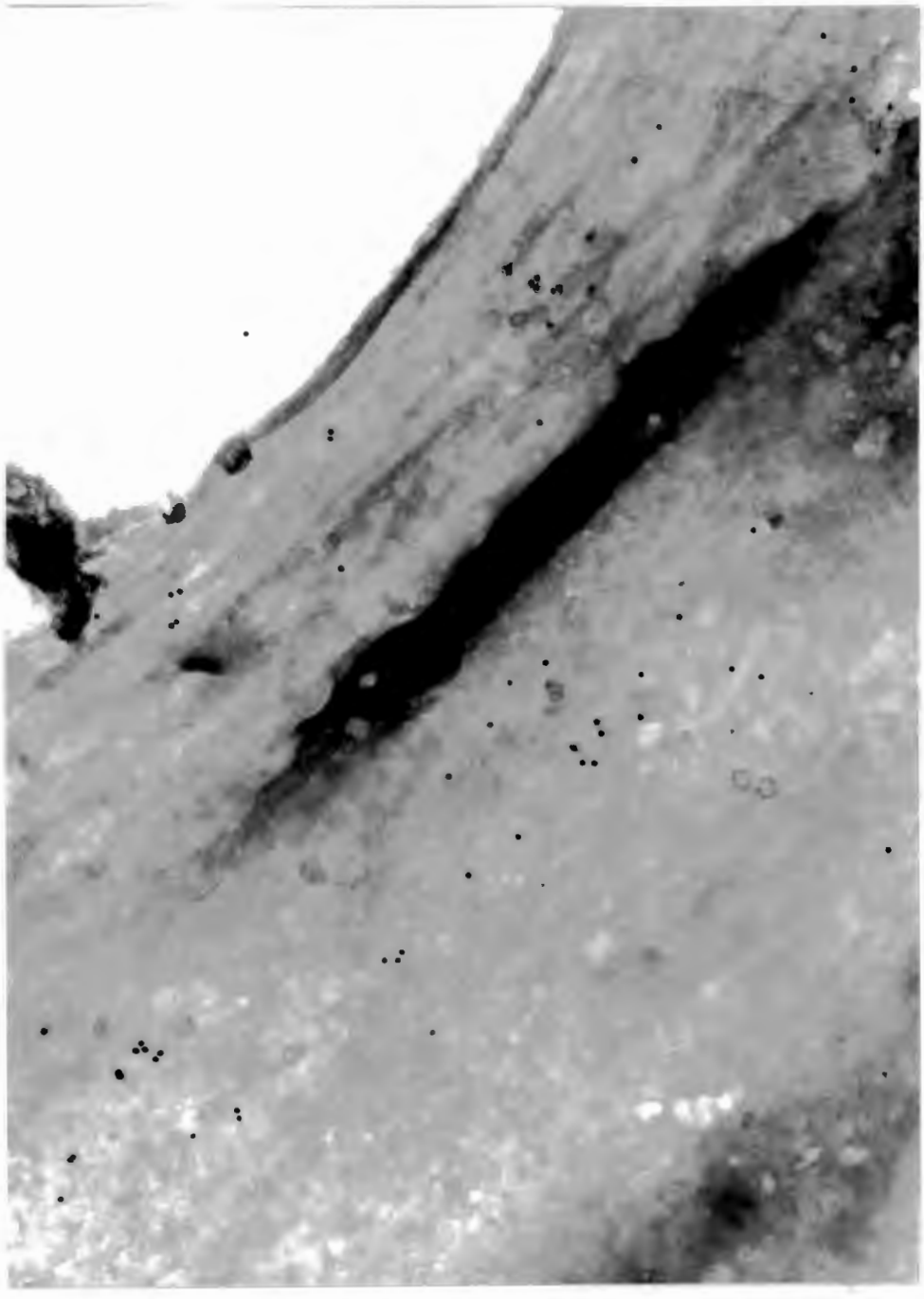


PLATE 4.3 A: Edge of a whole-mounted FGo cell probed at $4^{o}C$ with C7 antibodies which were subsequently labelled with colloidal gold-labelled anti-C7 antibodies. Whole-mount was air-dried and rotary-shadowed. The receptors are in loosely-organised groups which is probably a result of low-efficiency labelling by the indirect immunogold system. Dispersed receptors with no tendency to group were also seen. Similar cells labelled with Au-LDL (PLATE 4.6) showed grouped and clustered receptors, with less open space between the probes, indicating a higher efficiency of receptor marking with Au-LDL. Transmission electron micrograph. Magnification = 46 000x

Plate 4.3 B

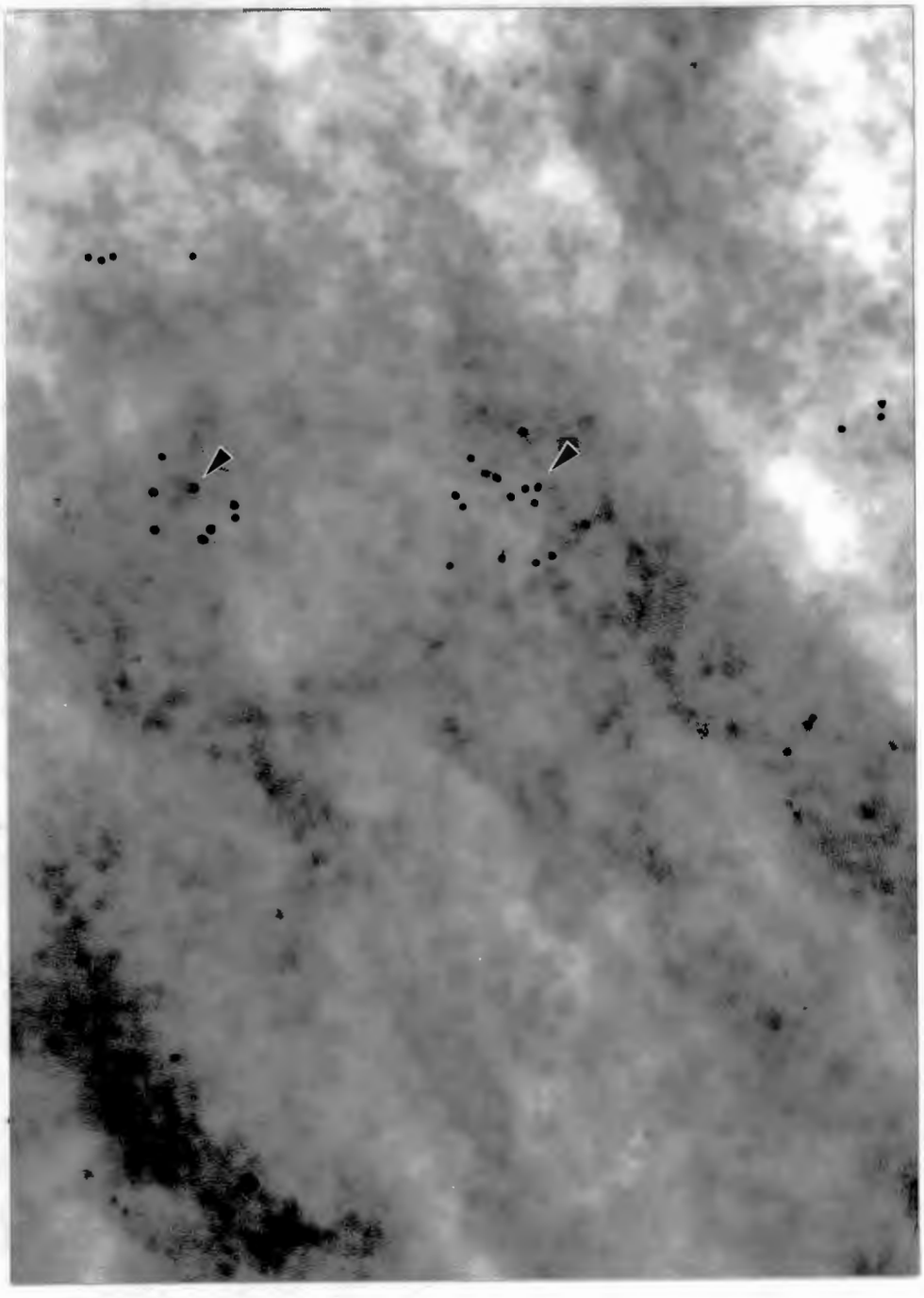


PLATE 4.3 B: Edge of an air-dried FGo cell otherwise prepared in the same way as that in PLATE 4.3 A. Note the clustered receptors. Two clusters (arrowheads) have receptors arranged loosely in rings, i.e. with no receptors in the central region of the delineated group. Transmission electron micrograph. Magnification = 72 000x

Plate 4.4 A, B

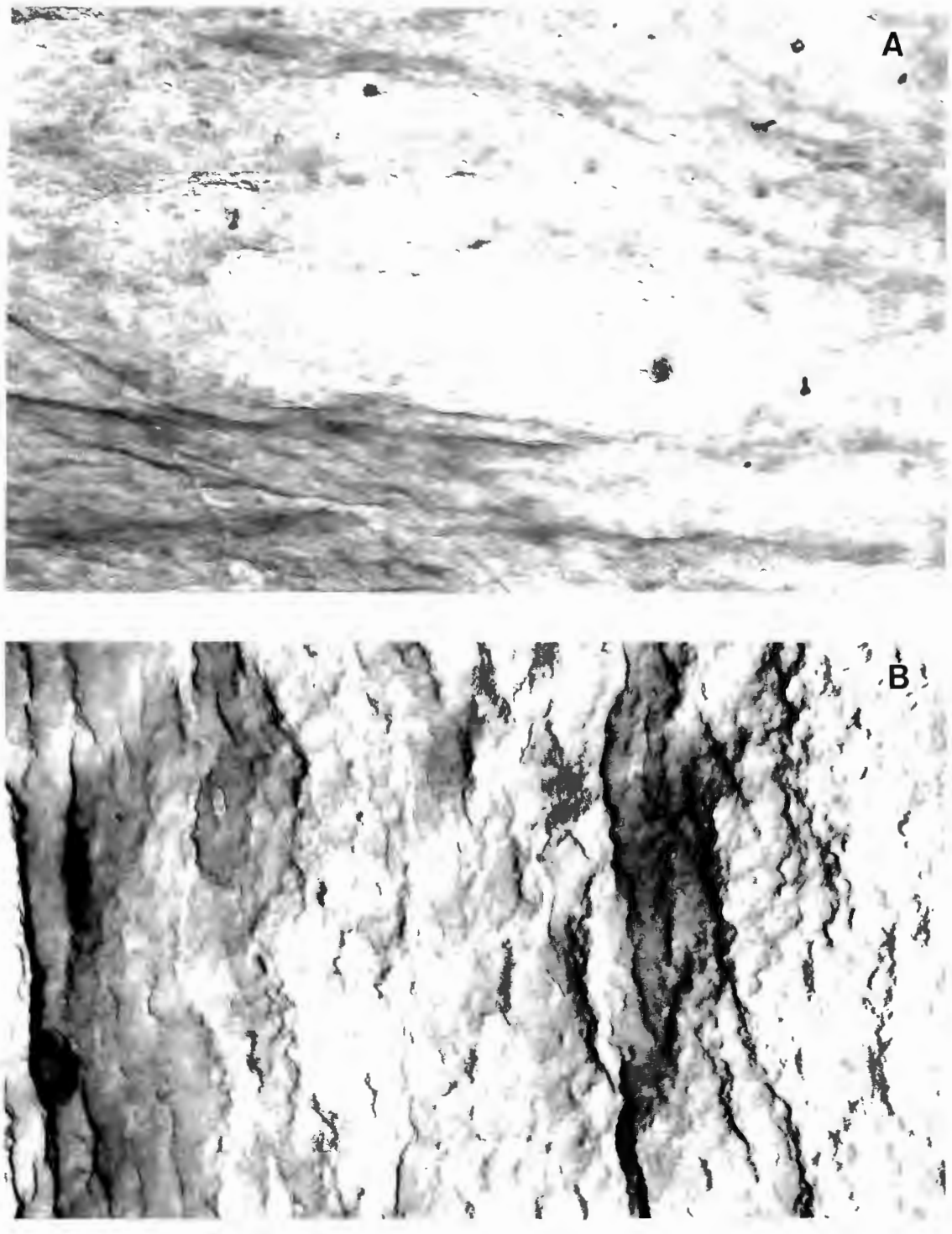


PLATE 4.4: Whole-mounted GM 2000 cells probed with C7 and GAM-G15. The cells were incubated with antibodies at 4°C, critical point dried and rotary-shadowed.
A: Low-magnification (9800x) micrograph of the cell surface which is devoid of gold particles.
B: Higher-magnification (60 000x) micrograph of part of the same field.

Plate 4.5

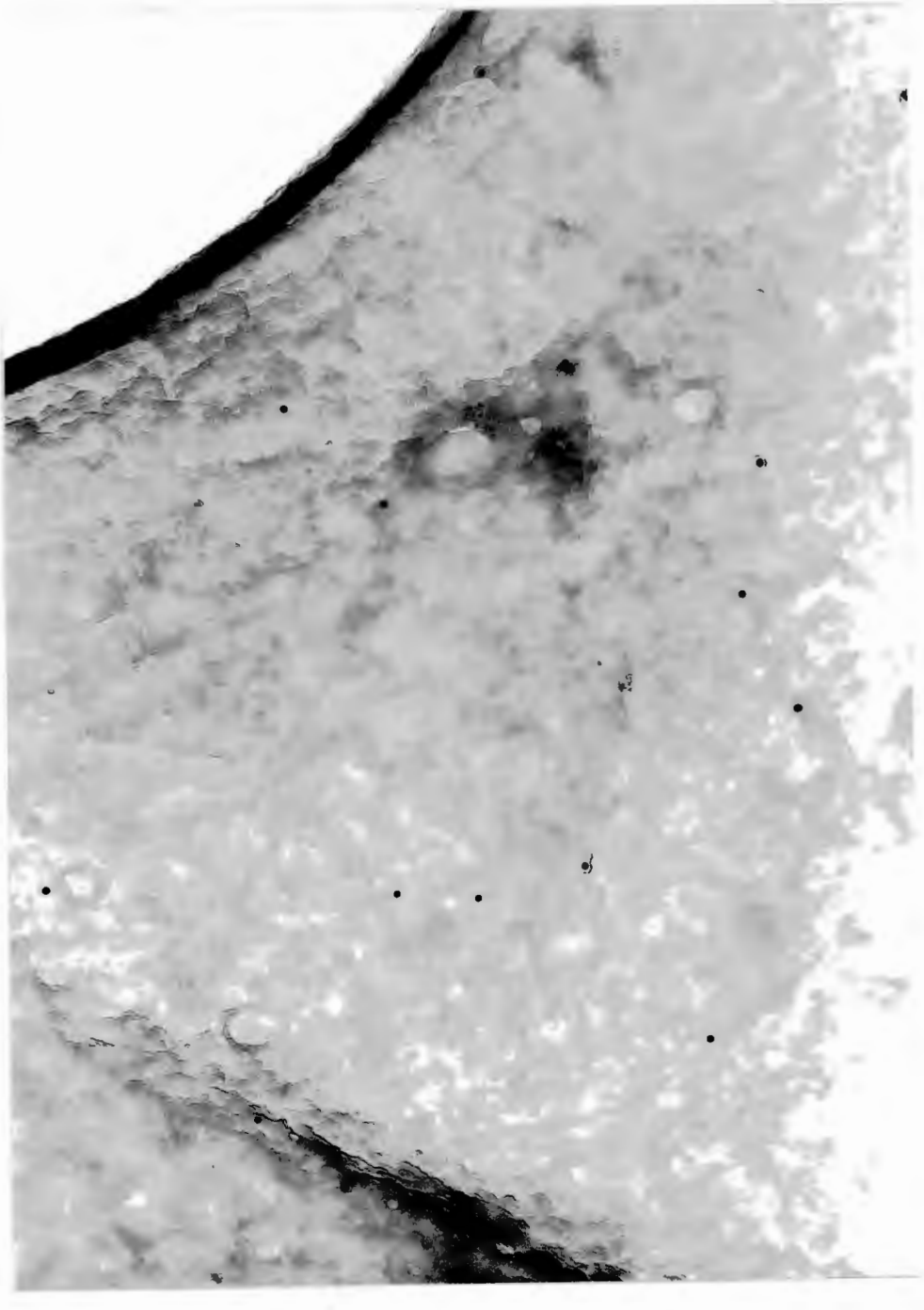


PLATE 4.5: The surface of a GM 2408A cell which has been incubated with C7 ($4^{o}C$) and GAM-G15. Sparsely distributed, mainly monodisperse gold particles are seen. Transmission electron micrograph. Magnification = 60 000x.

Plate 4.6 A

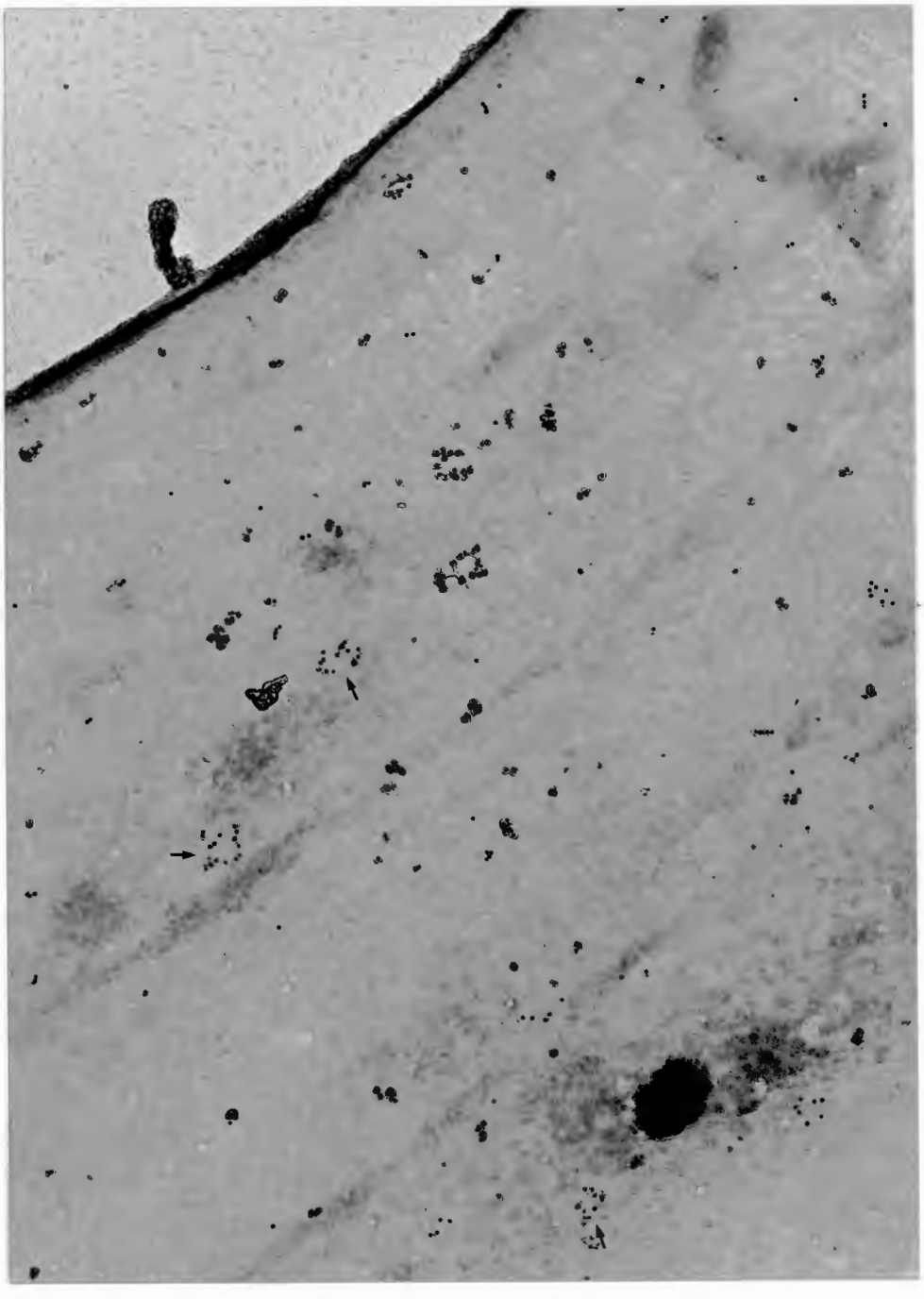


PLATE 4.6 A: FGo cell whose LDL-Rs have been marked with Au-LDL at $4^{o}C$. Cells were critical point-dried, shadowed and examined as whole-mounts in the TEM. Low magnification (27 000x) micrograph showing clustered and dispersed receptors near the edge of the cell. The clusters are mostly ring-shaped. The Au-LDL is lying in depressions in a number of regions as there are no haloes around the particles of gold (arrows).

Plate 4.6 B

PLATE 4.6 B: FGo cell as in PLATE 4.6 A, but one very tightly-organised cluster, possibly representing a deep coated pit on the verge of endocytosis, is visible (arrowhead). Monodisperse receptors are particularly clear here. Magnification = 27 000x.

Plate 4.6 C

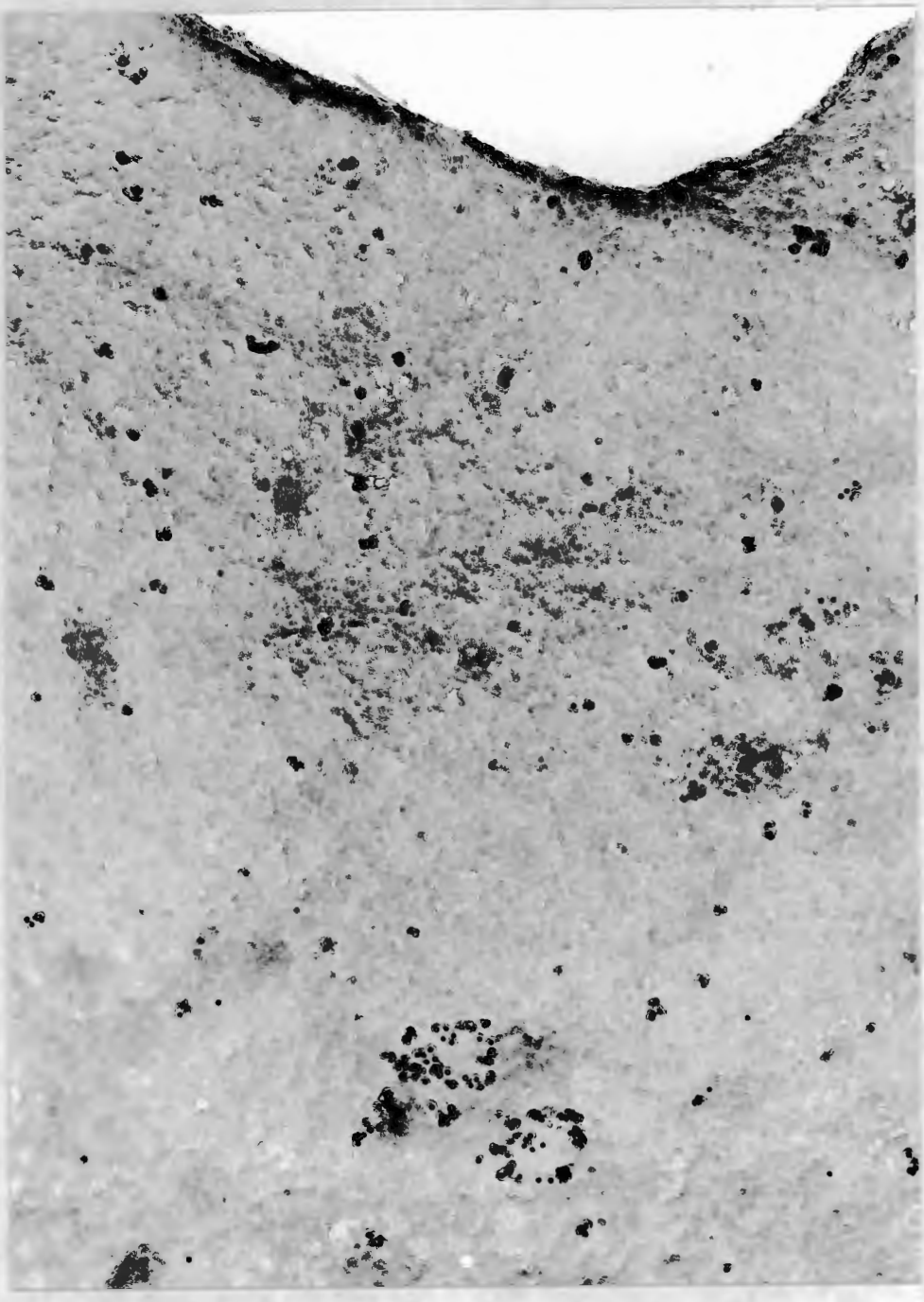


PLATE 4.6 C: FGo cell as in PLATE 4.6 A. Note the large number of dispersed receptors very close to the cell margin. Magnification = 37 000x.

PLATE 4.6 D

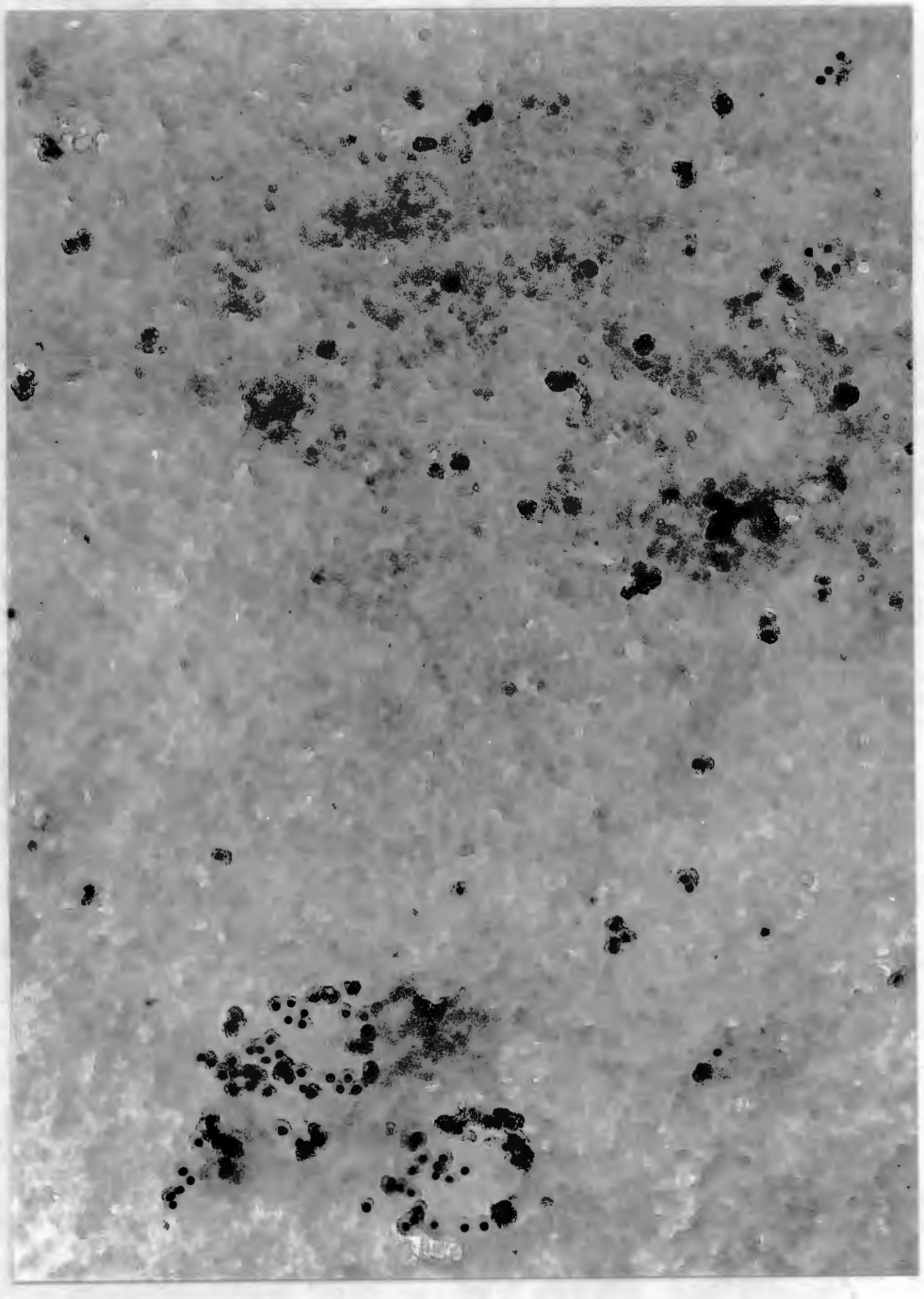


PLATE 4.6 D: FGo cell prepared as in PLATE 4.6 A. Enlarged area from PLATE 4.6 C showing double-ring cluster of receptors. Magnification = 60 000x.

Plate 4.7 A

PLATE 4.7 A: Micrographs of GM 2000 cells incubated at $4^{o}C$ with Au-LDL before preparation as whole-mounts. In this group of four fibroblasts, two cells (arrowed) have flattened marginal zones of the kind found suitable for the analysis of LDL-R distribution. Such marginal zones were used generally for all analyses reported in this chapter. The central parts of the cells were too thick for clear imaging of the upper surface ultrastructure in the TEM. Magnification = 6600x.

Plate 4.7 B

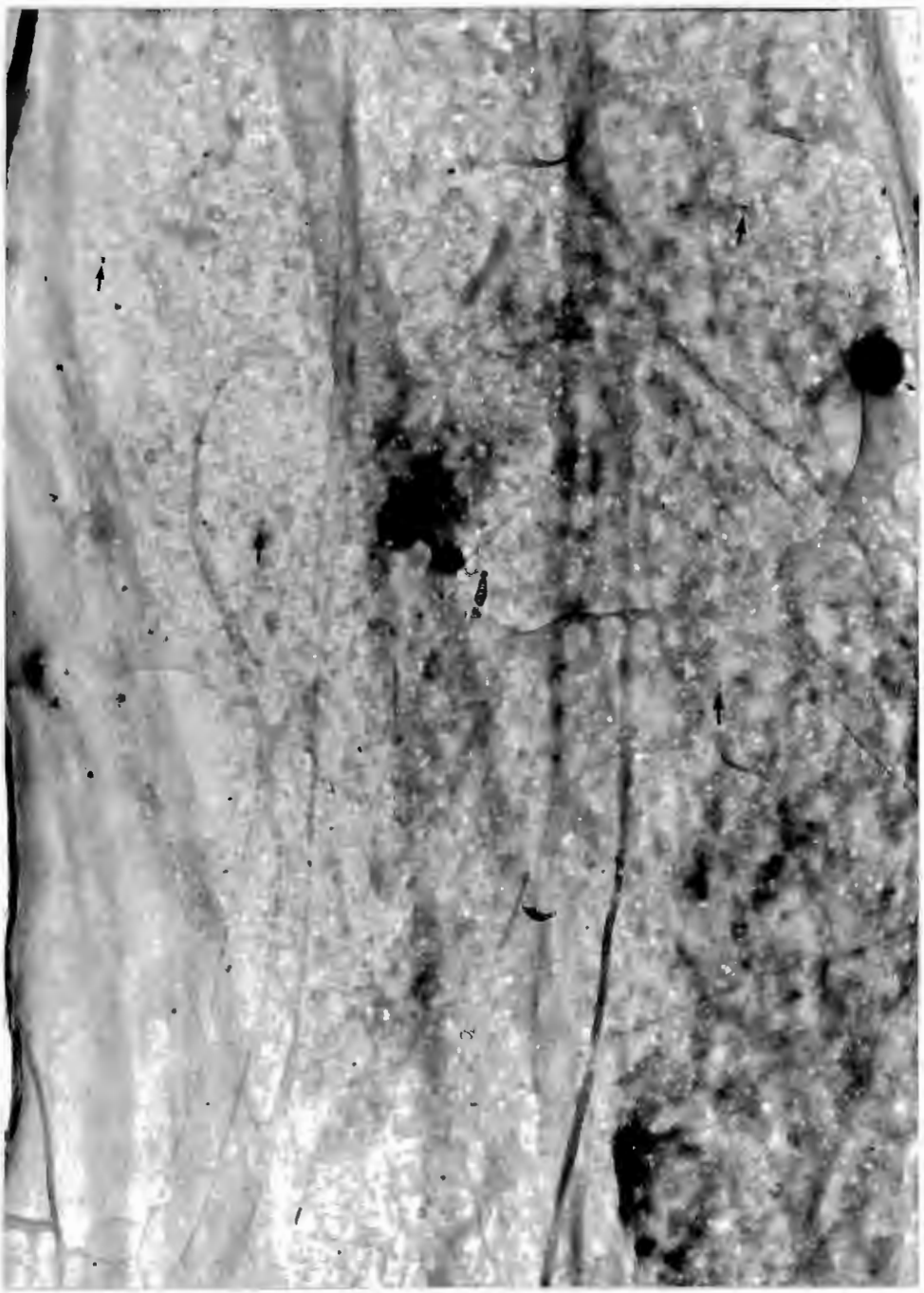


PLATE 4.7 B: Micrographs of GM 2000 cells incubated at 4°C with Au-LDL before preparation as whole-mounts. Medium magnification (16 400x) micrograph of part of a cell. Very few gold particles can be seen (arrows). This represents the background level of Au-LDL on fibroblasts.

Plate 4.7 C

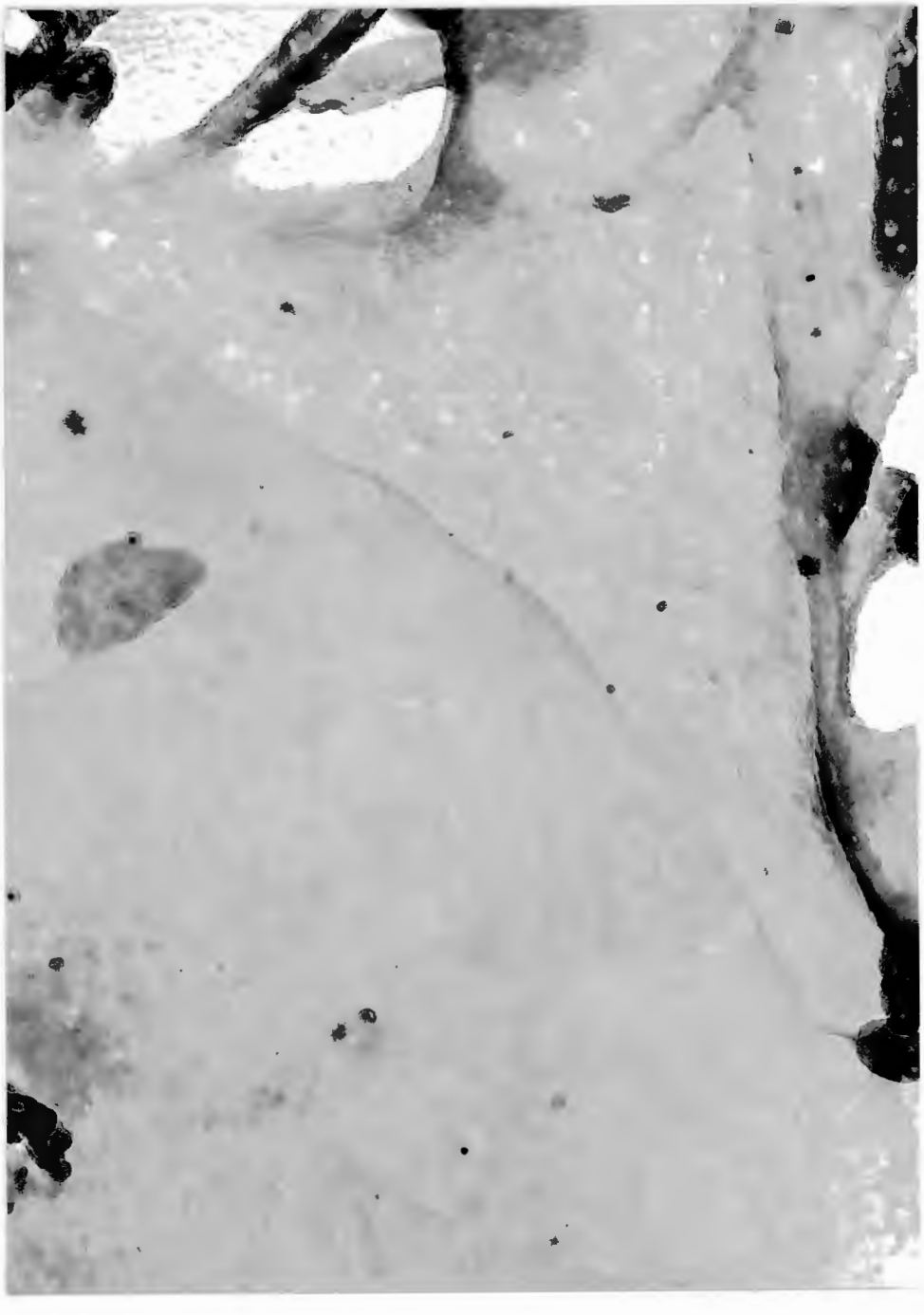


PLATE 4.7 C: Micrographs of GM 2000 cells incubated at 4^{o}C with Au-LDL before preparation as whole-mounts. High magnification (46 000x) micrograph depicting the smooth cell surface with very few gold particles bound to it.

Plate 4.8 A

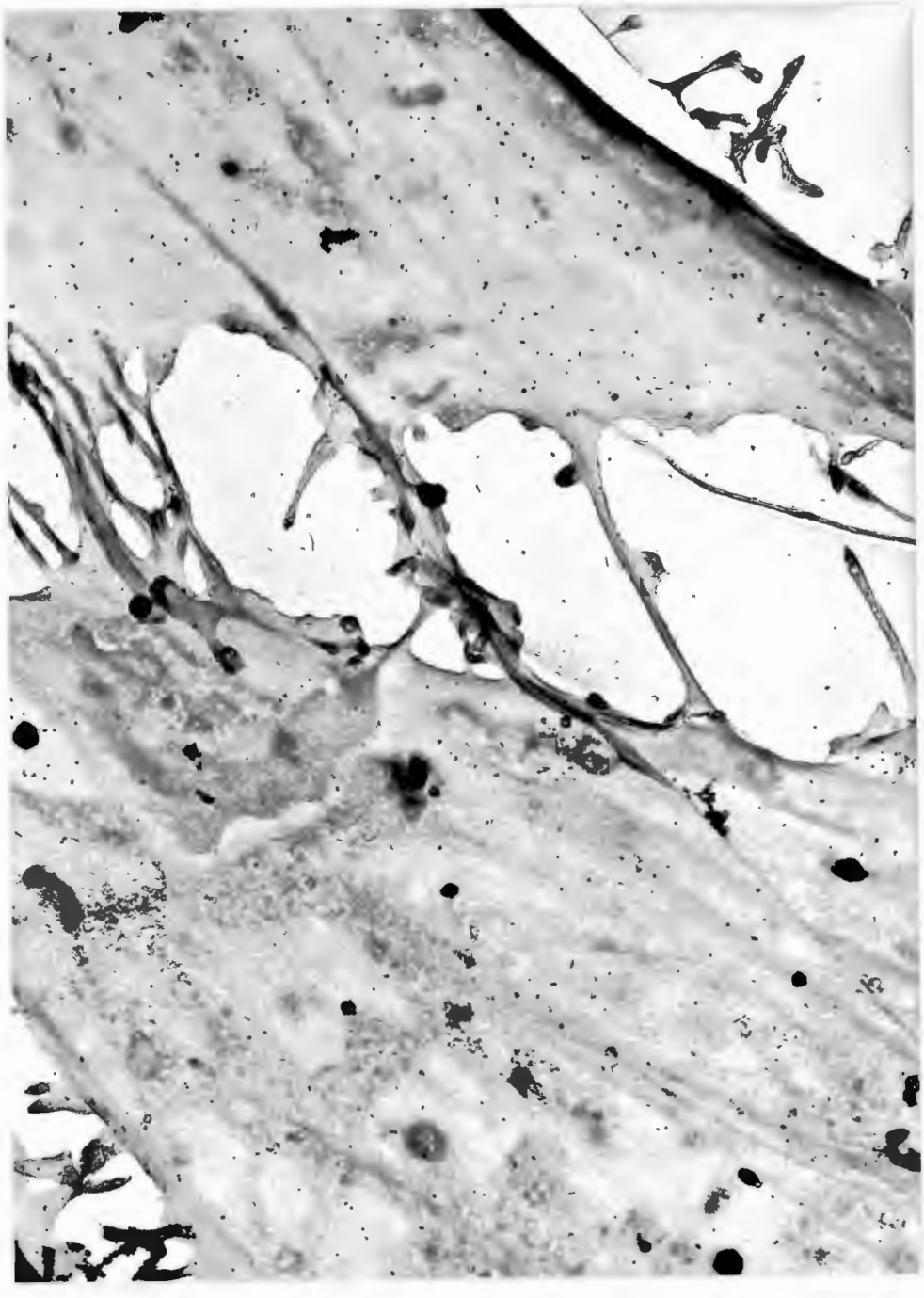


PLATE 4.8 A: Micrographs of GM 2408A cells incubated at $4^{o}C$ with Au-LDL and prepared as whole-mounts. Low magnification (9800x) micrograph depicting two cells which are conveniently flattened for the clear display of a large number of monodisperse receptors covering the cell surfaces in a random distribution.

Plate 4.8 B

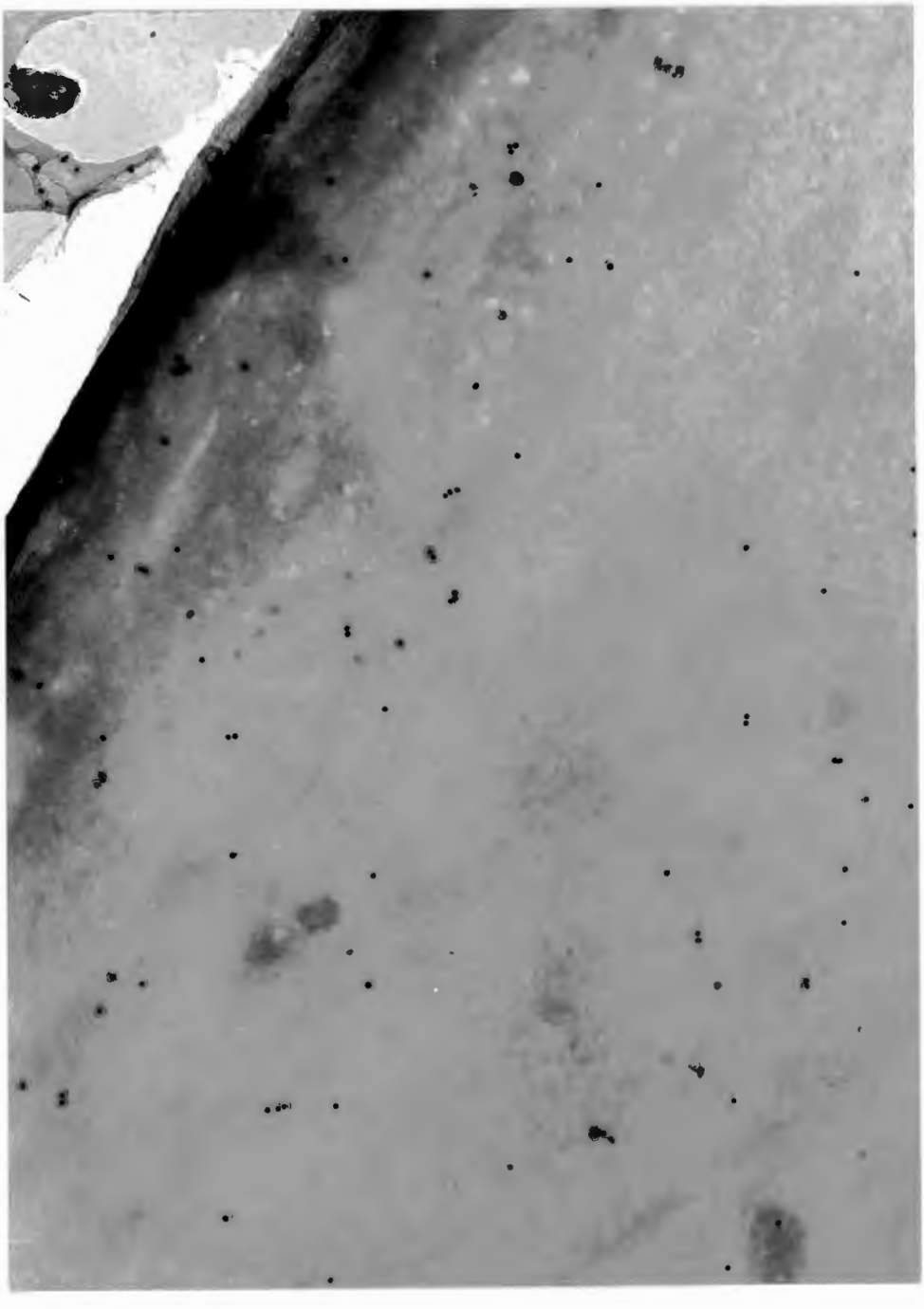


PLATE 4.8 B: Micrograph of GM 2408A cell incubated at $4^{o}C$ with Au-LDL and prepared as whole-mount. Medium magnification (37 000x) micrograph of the edge of a cell. Dispersed receptors are clearly seen.

Plate 4.8 C

PLATE 4.8 C: Micrograph of GM 2408A cell incubated at 4°C with Au-LDL and prepared as whole-mount. High magnification (46 000x) micrograph showing the disperse receptors labelled by gold probes. A few doublets, triplets and rare groups of four or five are also visible. A patch of carbon film representing the cast over the culture dish surface is completely free of probes (top).

Interaction of Au-LDL with Endothelial Cells

Following standardisation of the Au-LDL probe interaction with the LDL-R in fibroblasts, and cross-checking of the results by immunochemical mapping of LDL-R distribution, Au-LDL was used to map LDL-R in subconfluent and postconfluent bovine aortic ECs.

Subconfluent ECs

The Au-LDL pattern on subconfluent ECs resembled that found on FGo. Again, many ring-shaped clusters of Au-LDL, ranging in diameter from 200 to 500 nm, were seen (PLATE 4.9 A-G). They were often compound consisting of two or three coalescent rings (PLATE 4.9 B,E; PLATE 4.10 A,B). Separate rings occurred singly or in groups (PLATE 4.9 A-D). The plasma membrane encircled by the rings sometimes appeared smoother than the surrounding areas. Where thicker regions of cells were imaged, the area upon which the Au-LDL was bound appeared depressed, like a shallow pit. This effect was present, but not so pronounced, in flatter regions of the cell where the cytoplasmic layer was thin. A monodisperse population of Au-LDL probes, similar to that seen on FGo cells, was also detected on subconfluent ECs. These diffuse or scattered receptors occurred amongst the ring-shaped clusters (PLATE 4.9 A-G). PLATE 4.9 G depicts an area of the cell surface close to the margin of a spreading cell: here the scattered receptors appear alone, without neighbouring clusters. As in fibroblasts, the labelling was not uniform from region to region on a cell surface, nor from cell to cell within an ECs whole-mount preparation. Contiguous cells often differed greatly in the extent to which they bound the Au-LDL probe. One cell might be heavily labelled while its neighbour was almost free of probes. Besides being an excellent internal control for non-specific binding, this illustrated the variability of expression of the LDL-R on individual cells within a culture. Similar variations in receptor expression from cell to cell were seen in normal fibroblasts as mentioned before. To give an idea of the variety of cluster shapes and sizes seen, a gallery of micrographs is presented (PLATE 4.10 A-C). The individual Au-LDL probes can be resolved clearly in these higher magnification micrographs. The rotary shadowing with platinum-carbon has revealed the outlines of the LDL molecules surrounding the colloidal gold cores (PLATE 4.10 A,B). The gold cores are surrounded by lobed haloes resembling the Au-LDL probe as seen by negative staining (PLATE 4.2).

Some of the subconfluent E8 cultures which had been incubated with Au-LDL were embedded in resin, sectioned and examined in the TEM. The cross-sections revealed Au-LDL probes bound in coated areas of the plasma membrane which resembled coated pits. Probes in pits were usually in groups of three or more. A multiple or compound pit is illustrated in PLATE 4.11B where two closely adjacent pits each containing three probes are neighbours to an empty invaginated pit connected to the surface via a narrow neck. Immediately next to this is another shallow pit containing three probes. The arrangement of probes in rings around the edges of pits was confirmed in sectioned material where preferential labelling of pit edges was sometimes seen (PLATE 4.11 A,C). If an ultrathin section is assumed to be 75 nm thick, then 4 - 5 sections will account for an average pit 350 nm in diameter. If such a pit contained 15 probes arranged circumferentially, then a section across the middle might contain two probes while sections across and including the edges might contain as many as six probes. The actual numbers of probes seen in sections were consistent with these theoretical projections. Occasionally single probes not bound to coated regions of the plasma membrane were seen in sections, but only rarely. Intracellular probes were not seen which was consistent with arrest of the endocytotic process by incubation at 4^{0}C.

Plate 4.9 A

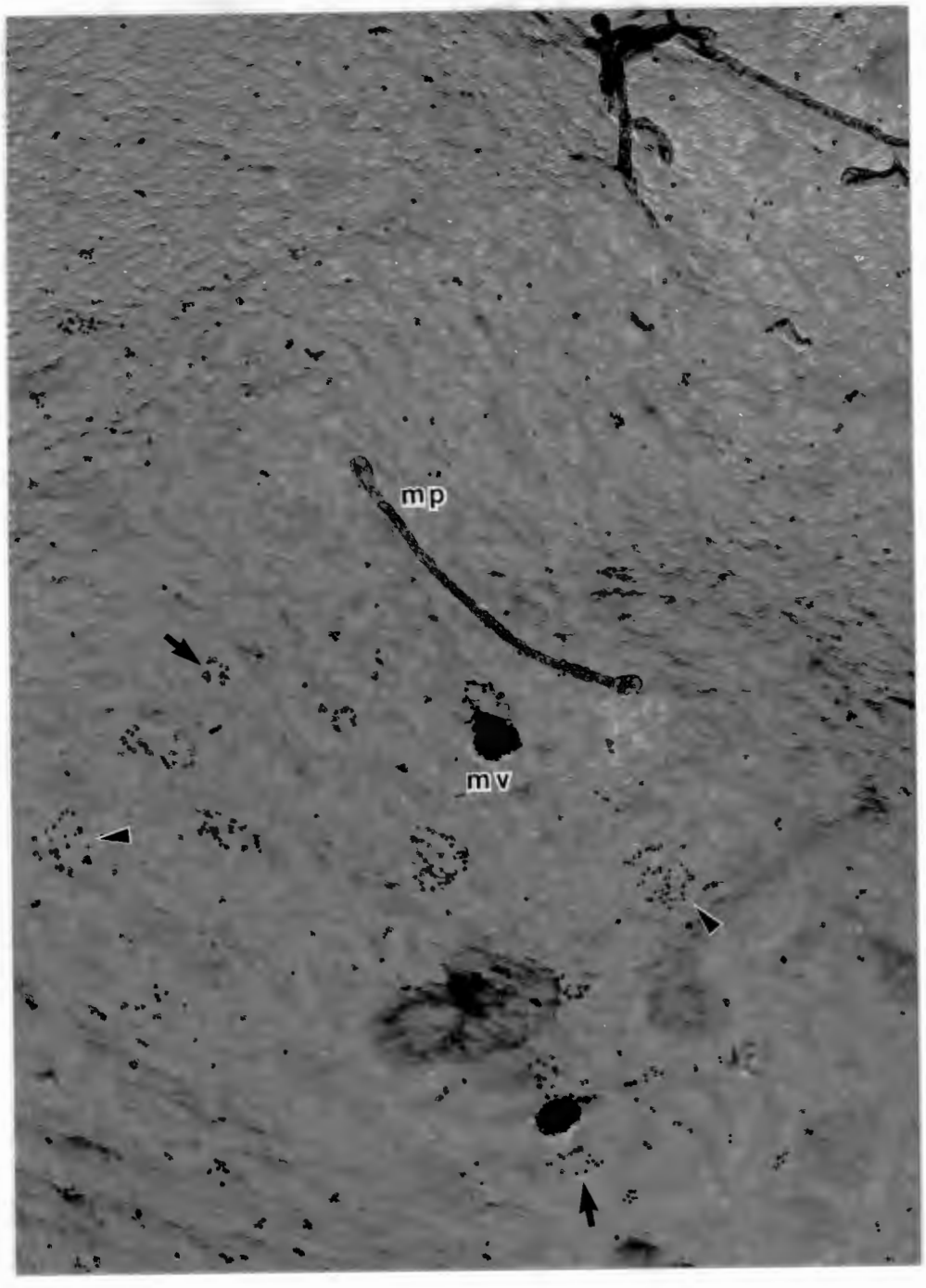


PLATE 4.9 A: Actively spreading subconfluent E8 endothelial cell which has been incubated with Au-LDL at 4°C. Receptors in large compound ring-clusters (arrowheads), smaller ring-clusters (arrows) and monodisperse receptors were intermingled on the EC cell surface. Some microprocesses (mp) and microvilli (mv) are present. Transmission electron micrograph. Magnification = 21 000x.

Plate 4.9 B

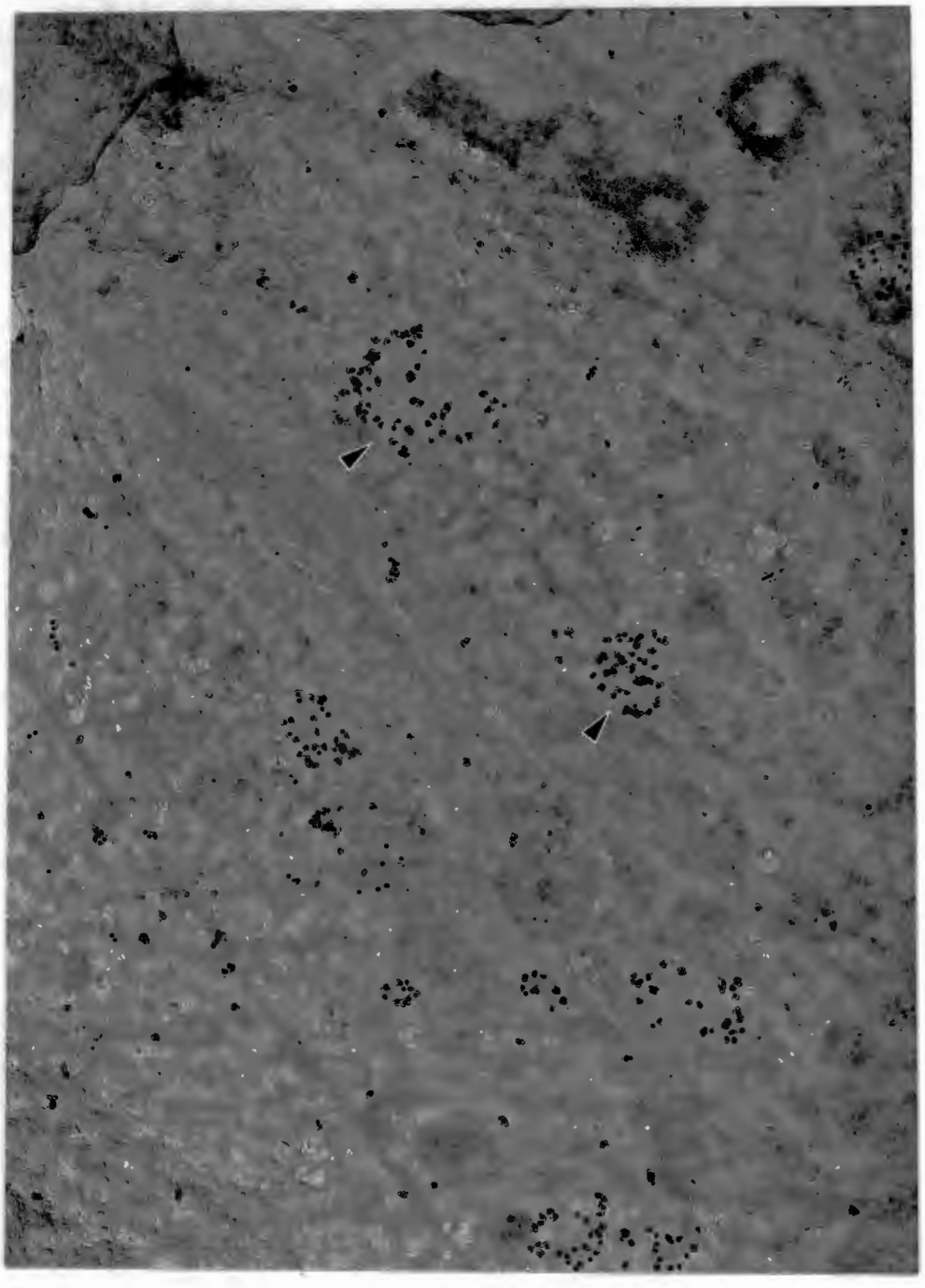


PLATE 4.9 B: Actively spreading subconfluent E8 endothelial cell which has been incubated with Au-LDL at 4°C. Groups of small ring-clusters and compound clusters (arrowheads) of receptors intermingled with monodisperse receptors near the cell margin. It appears that there are more monodisperse receptors nearer the cell margin. Magnification = 27 000x.

Plate 4.9 C

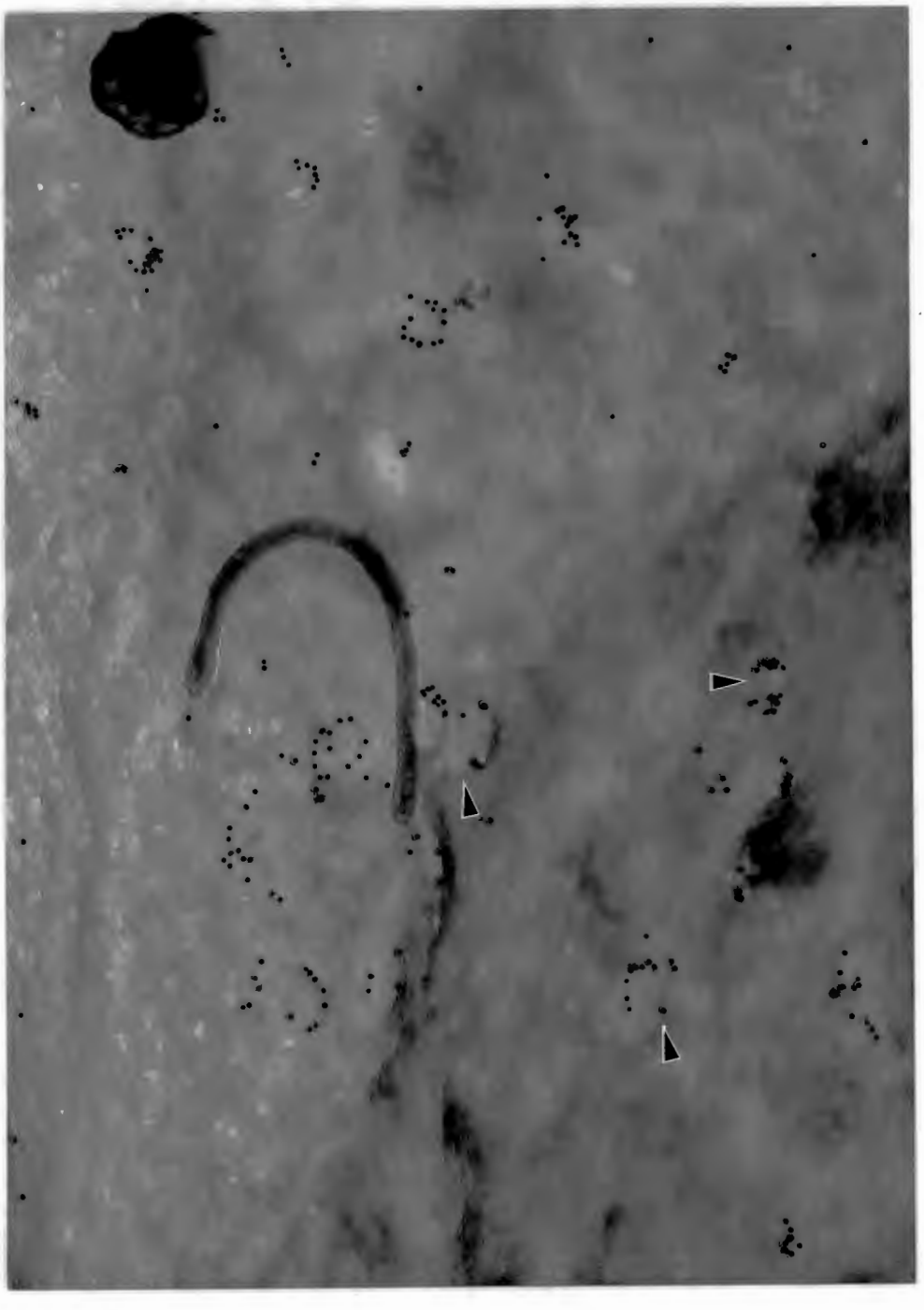


PLATE 4.9 C: Subconfluent E8 endothelial cell incubated with Au-LDL at 4^{o}C. Image of a thicker portion of a cell (away from the thinner marginal regions) which shows grouped ring-clusters delineating pit-like depressions (arrowheads) in the plasma membrane. Magnification = 37 000x.

Plate 4.9 D

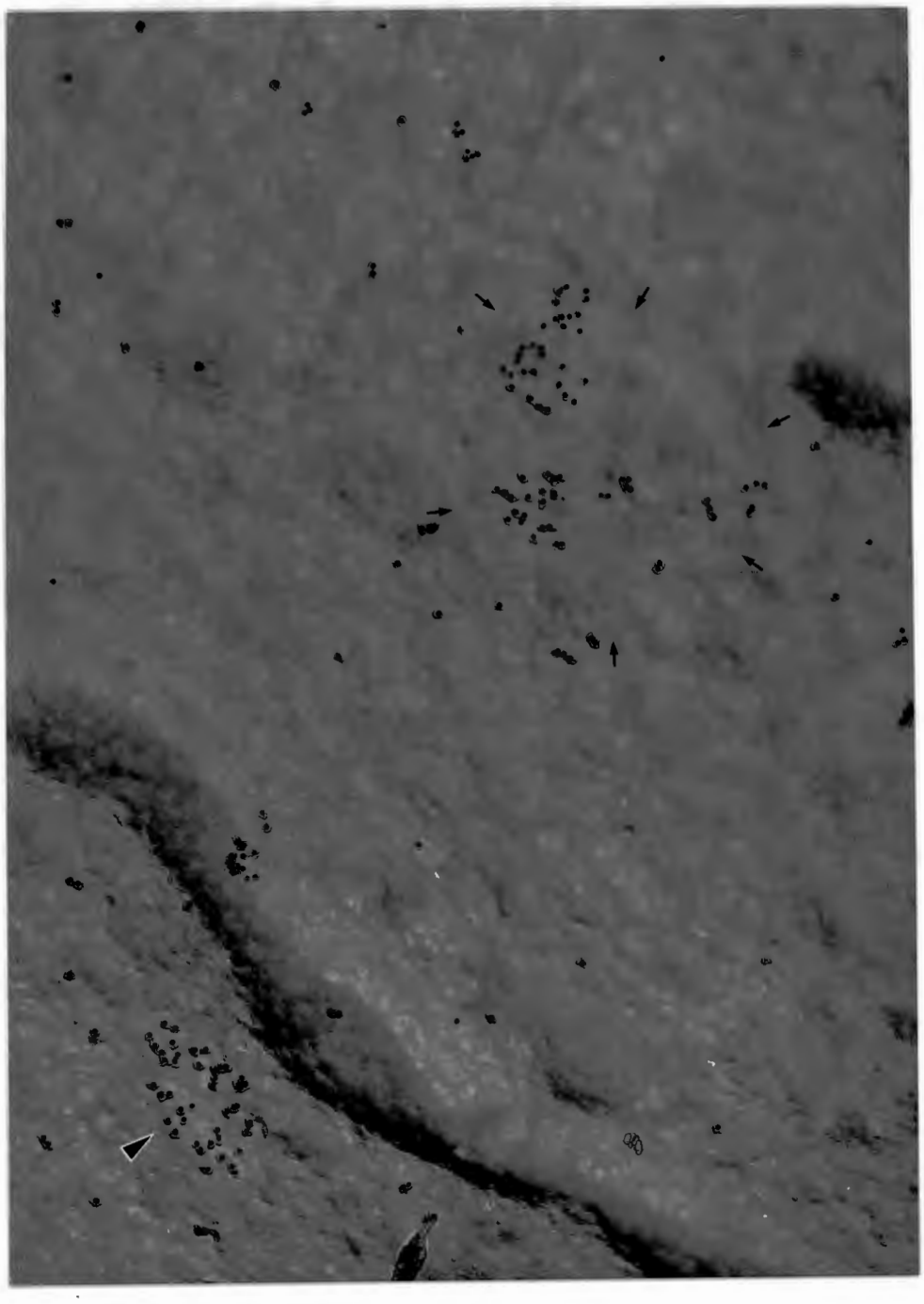


PLATE 4.9 D: Subconfluent E8 endothelial cell incubated with Au-LDL at 4°C. Two groups of receptors representing stages in the formation of coated pits.
Small arrows = loosely-associated receptors; arrowheads = more tightly-associated group.
Magnification = 37 000x.

Plate 4.9 E

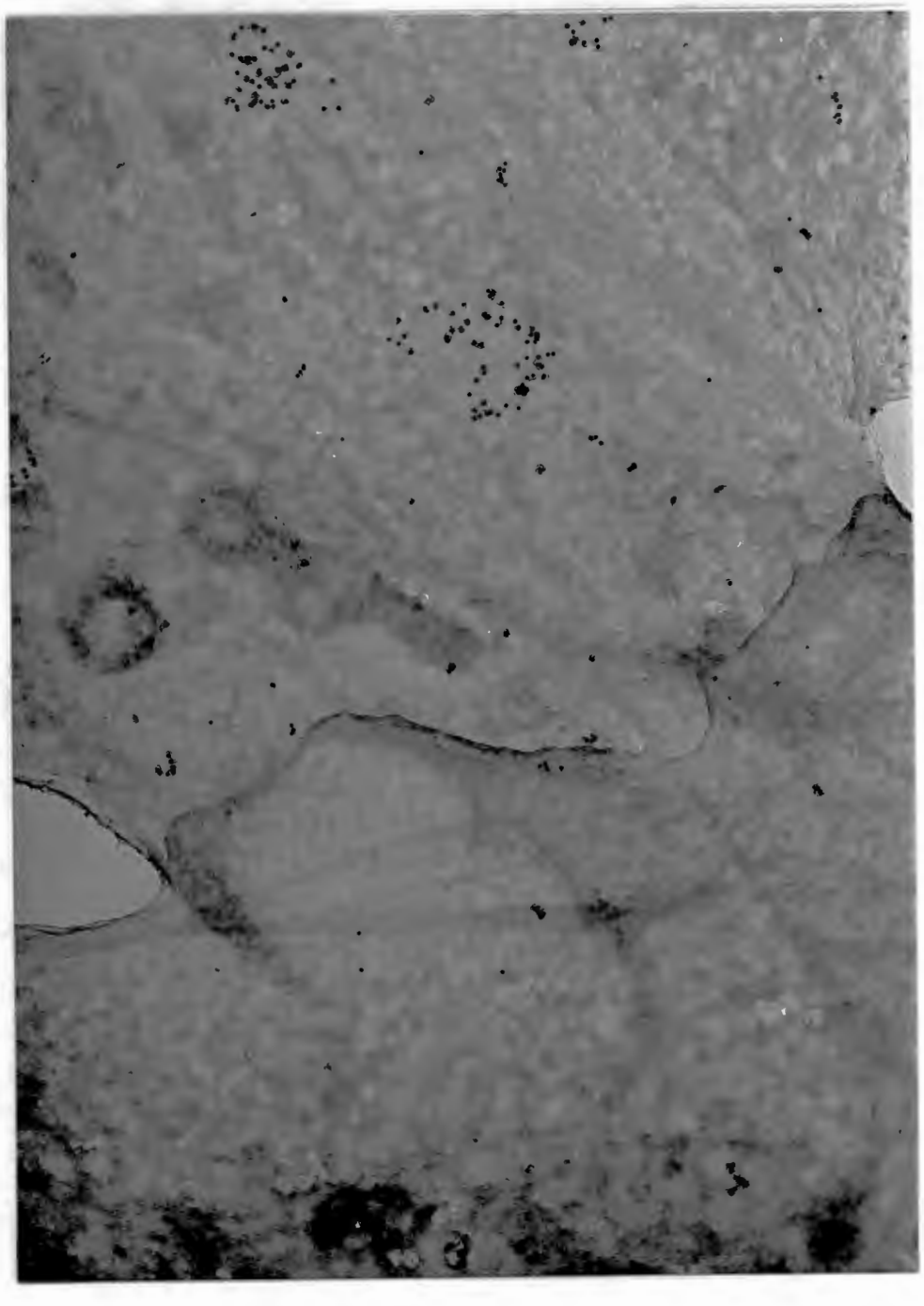


PLATE 4.9 E: Subconfluent E8 endothelial cells incubated with Au-LDL at 4^{o}C. Contiguous cells: the upper one has more receptors than the lower. Note the variations in cluster shape in the upper cell. Magnification = 27 000x.

Plate 4.9 F

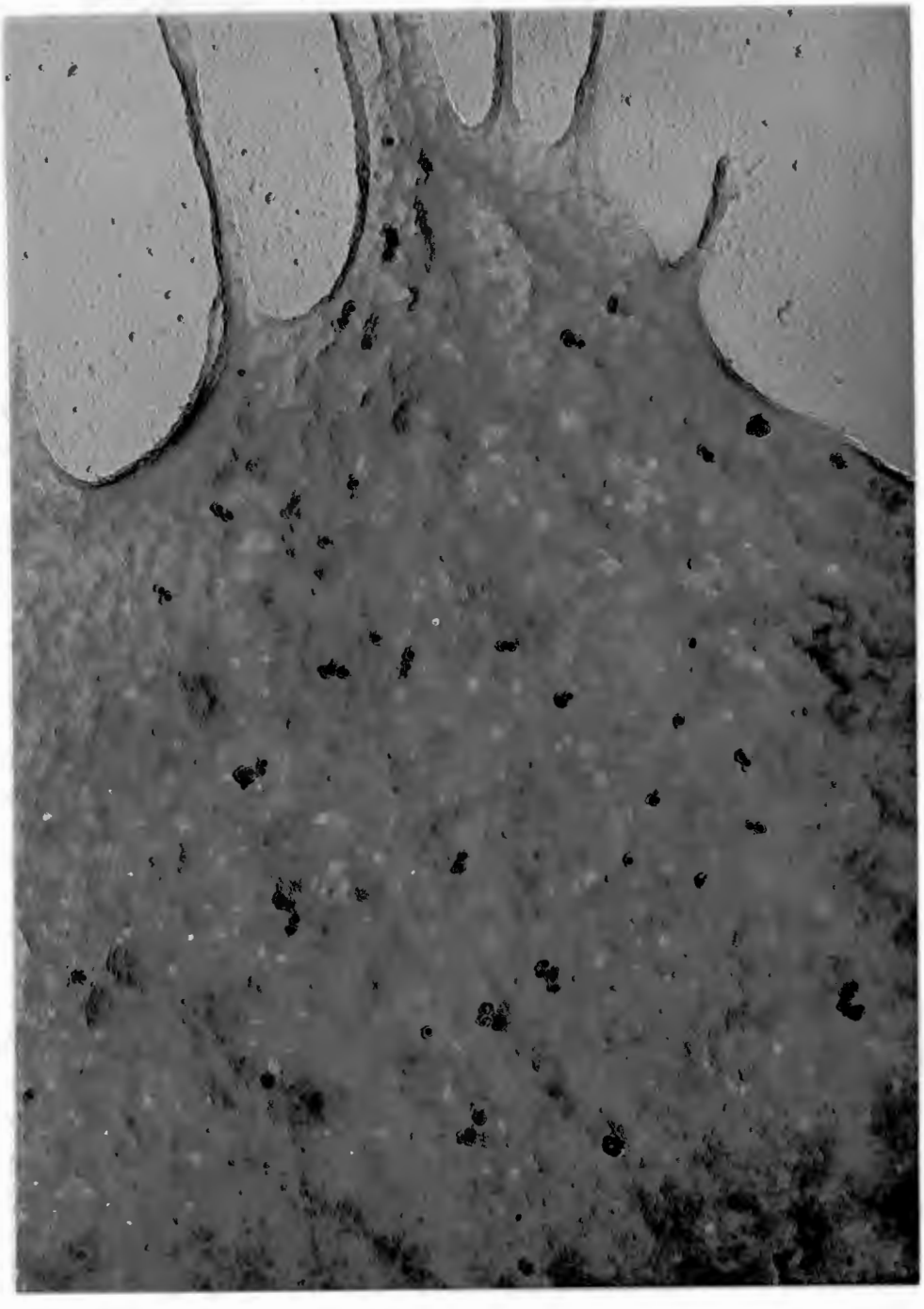
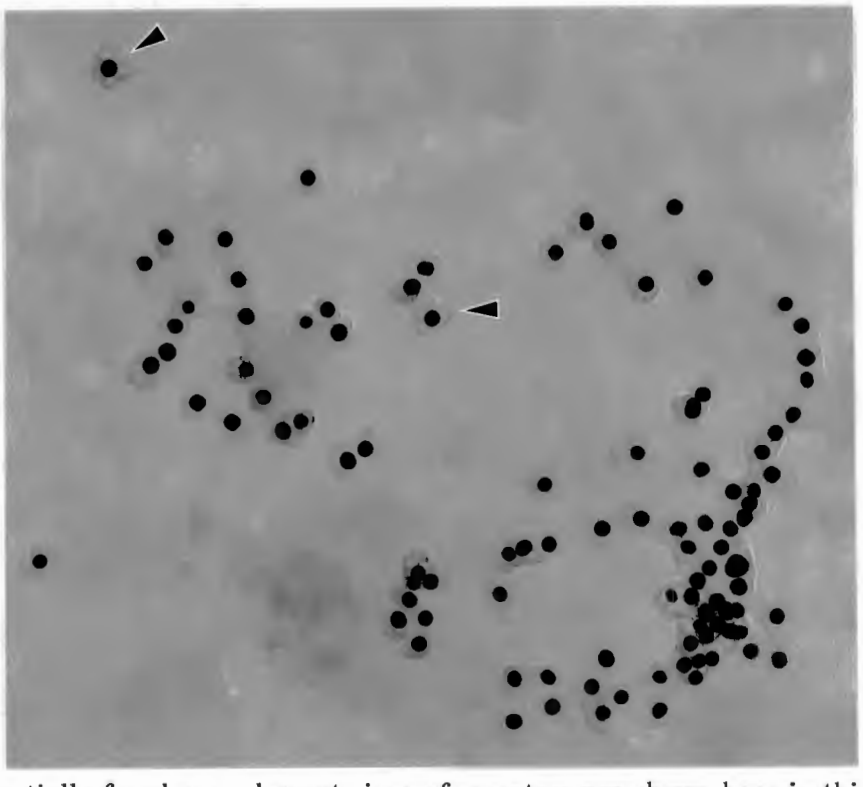


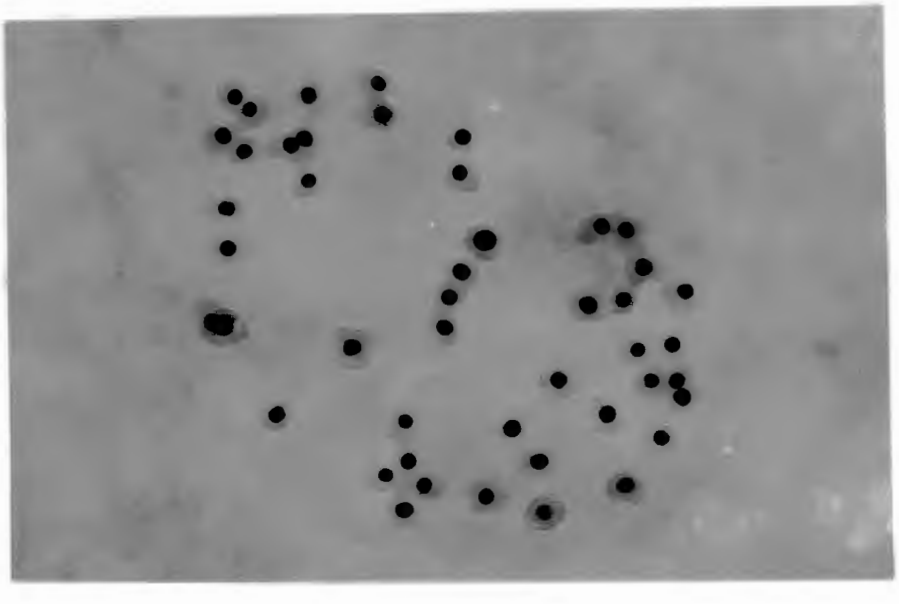
PLATE 4.9 F: Subconfluent E8 endothelial cell incubated with Au-LDL at 4^{o}C. Dispersed receptors at the extreme margin of cell. Magnification = 46 000x.

Plate 4.10 A - C

The following sequence of three micrographs (A-C) shows four coated pits of decreasing size on subconfluent E8 cells. It has been chosen to suggest the progressive association of receptors into clusters and the contraction of these clusters into tightly-packed endocytic pits. Magnifications (A - C) = 120 000x.



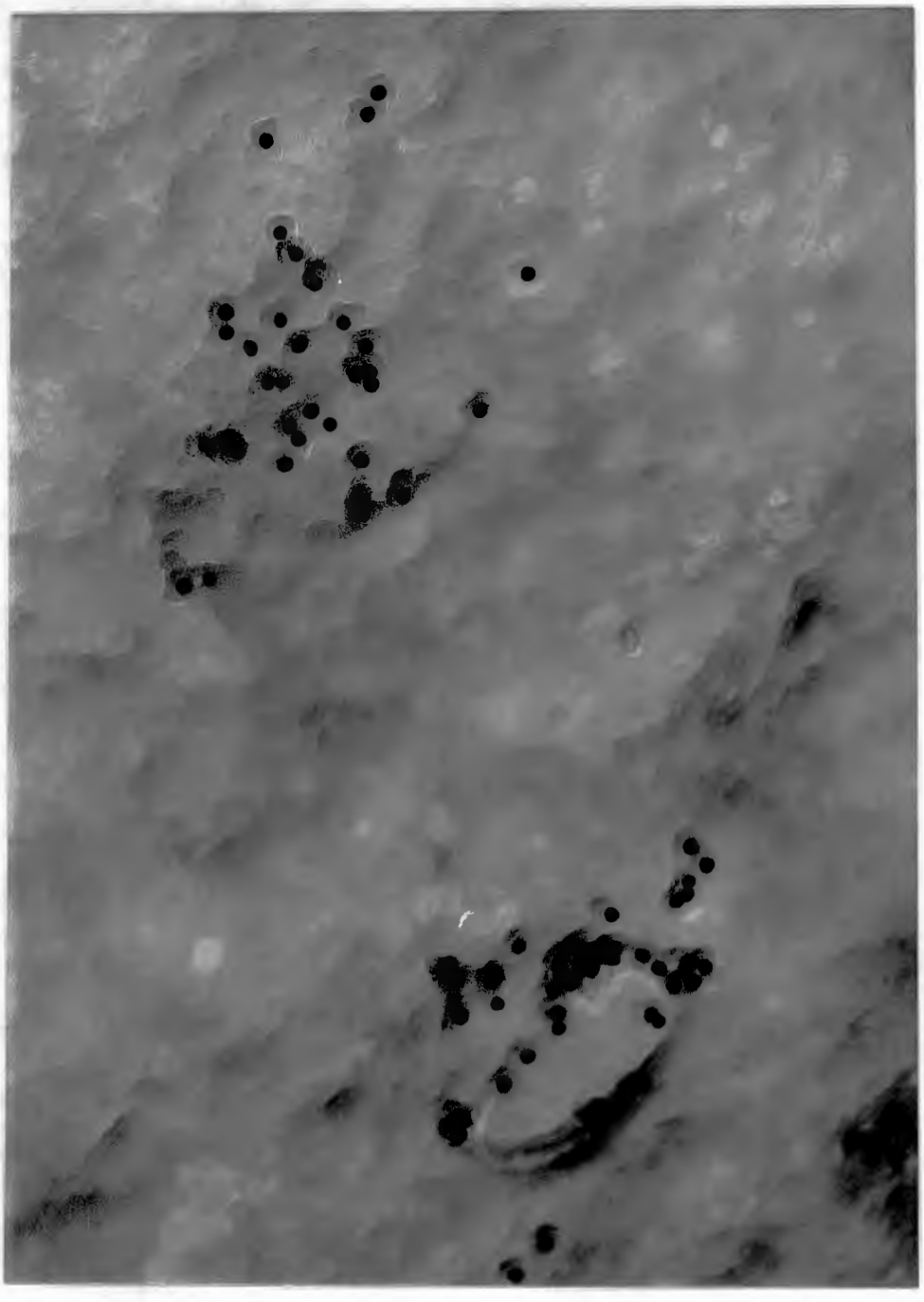
A: Several partially fused or coalescent rings of receptors are shown here in this compound ring-cluster. Such large compound clusters represent associations of several coated pits. Lobed outlines reminiscent of LDL molecules forming the outer shell of the Au-LDL probe can be seen. Lighter globular regions representing LDL molecules can be seen within the coating haloes (arrowheads).



B: Two fused ring-clusters, each of the order of 500 nm in diameter, are seen here. These probably represent two coated pits in very close proximity, or even fused.

Plate 4.10 C

Continues the sequence started in Plate 4.10 A.



C: The upper cluster represents a group of receptors in a more contracted coated pit which may be starting to invaginate. Below it, a fully contracted cluster of receptors appears to decorate the lip of a coated pit which is clearly invaginating in its progress towards becoming an endocytic vesicle. Magnification = 120 000x.

Plate 4.11 A

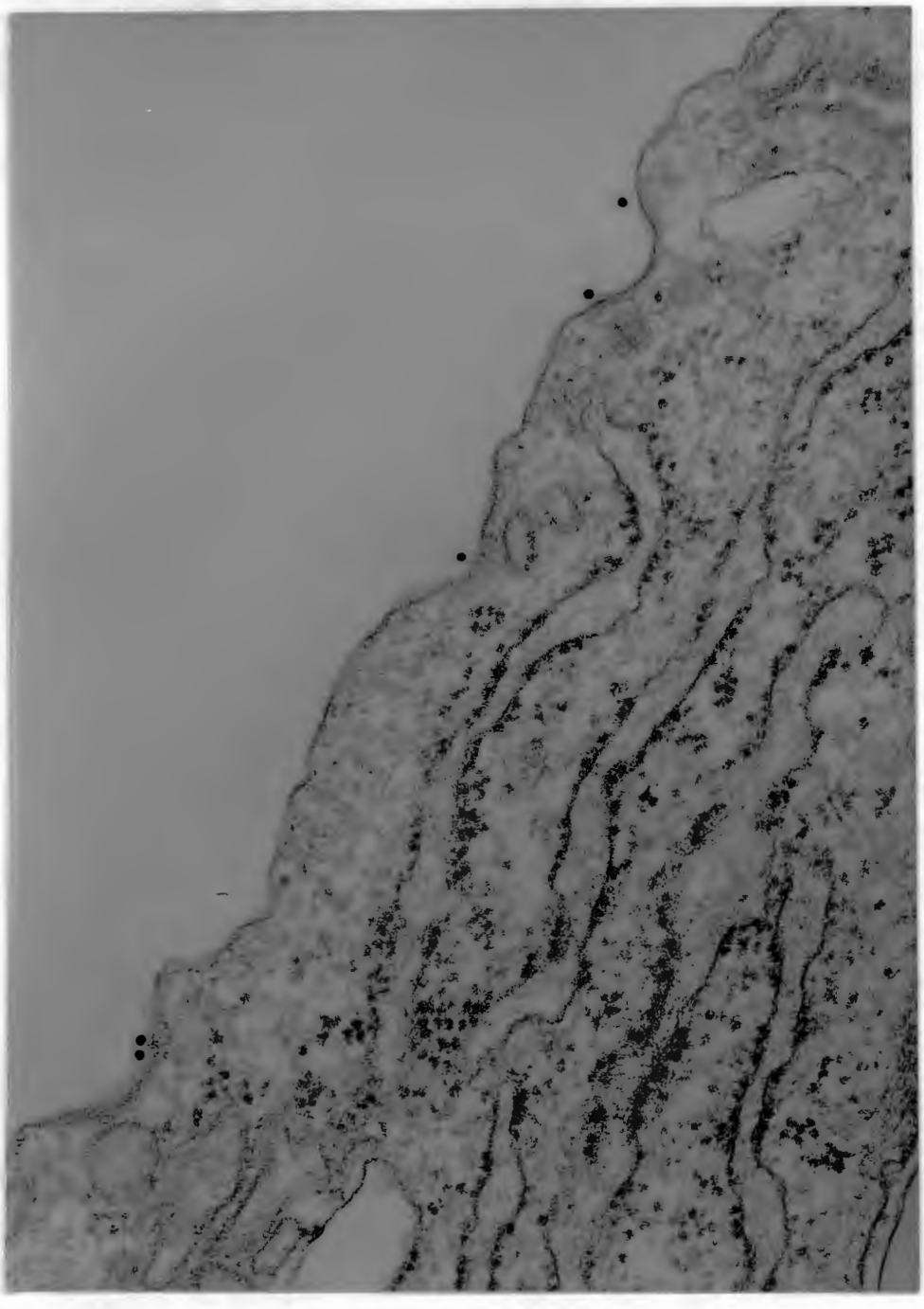


PLATE 4.11 A: Ultrathin section of an active, subconfluent E8 cell culture incubated with Au-LDL at 4°C before processing for EM. The "abluminal" or basal front of the cell is on the right. Two adjacent coated pits each containing Au-LDL probes are seen. The pit containing two peripherally bound probes has a fuzzy coating which is clearly visible on the inner surface of the transversely sectioned plasma membrane. Magnification = 72 000x.

Plate 4.11 B

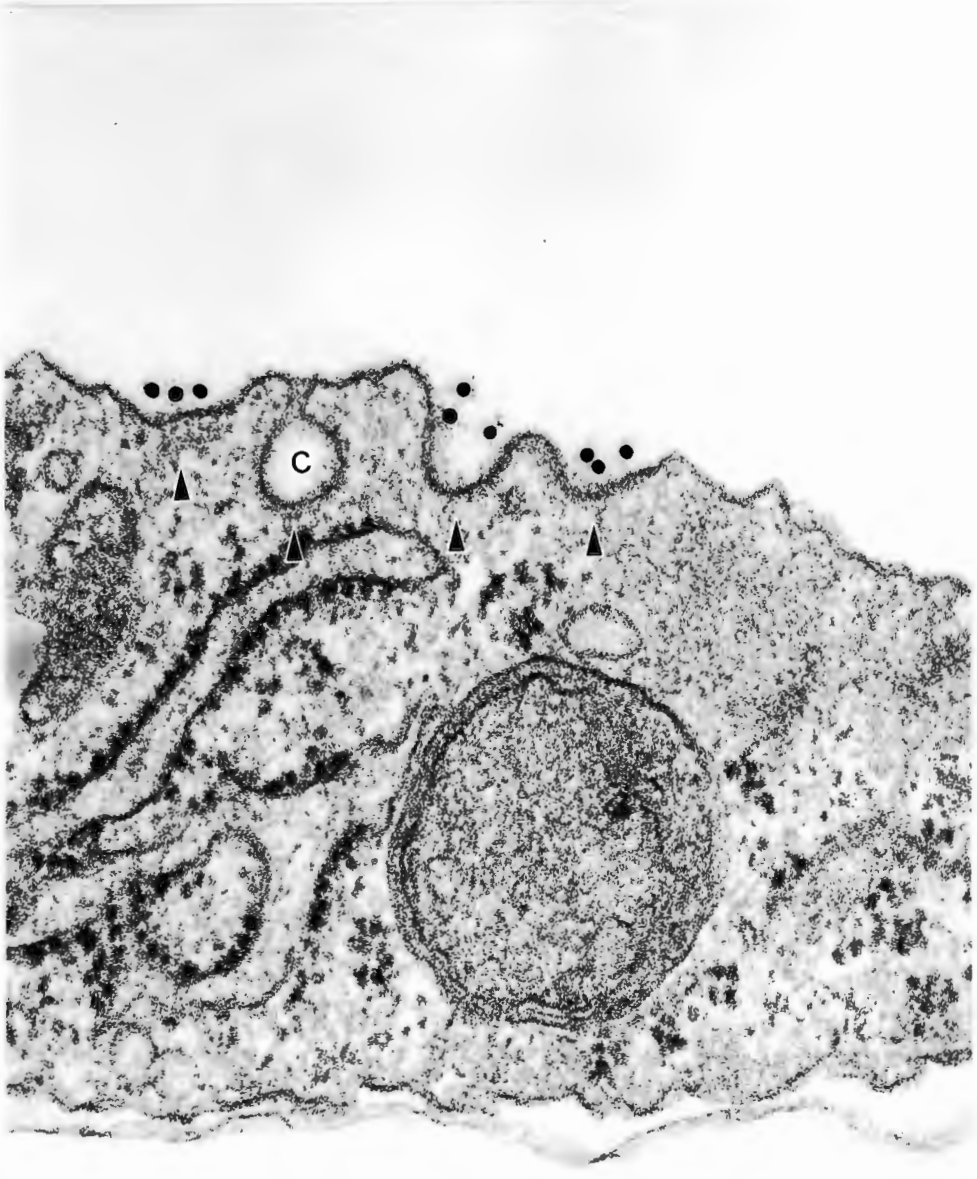


PLATE 4.11 B: Ultrathin section of an active, subconfluent E8 cell culture incubated with Au-LDL at 4°C before processing for EM. The "abluminal" or basal front of the cell is at the bottom. There is no basement membrane as the cultures were not yet mature. Four adjacent coated structures of increasing depth are seen (arrowheads). Three of the pits are open on the apical front of the cell and are labelled with Au-LDL probes. The fourth coated structure (c) is almost a coated vesicle communicating with the surface via a narrow neck. Even if there were LDL-Rs present in this flask-shaped pit, Au-LDL could not penetrate it to label them. Magnification = 98 000x.

Plate 4.11 C

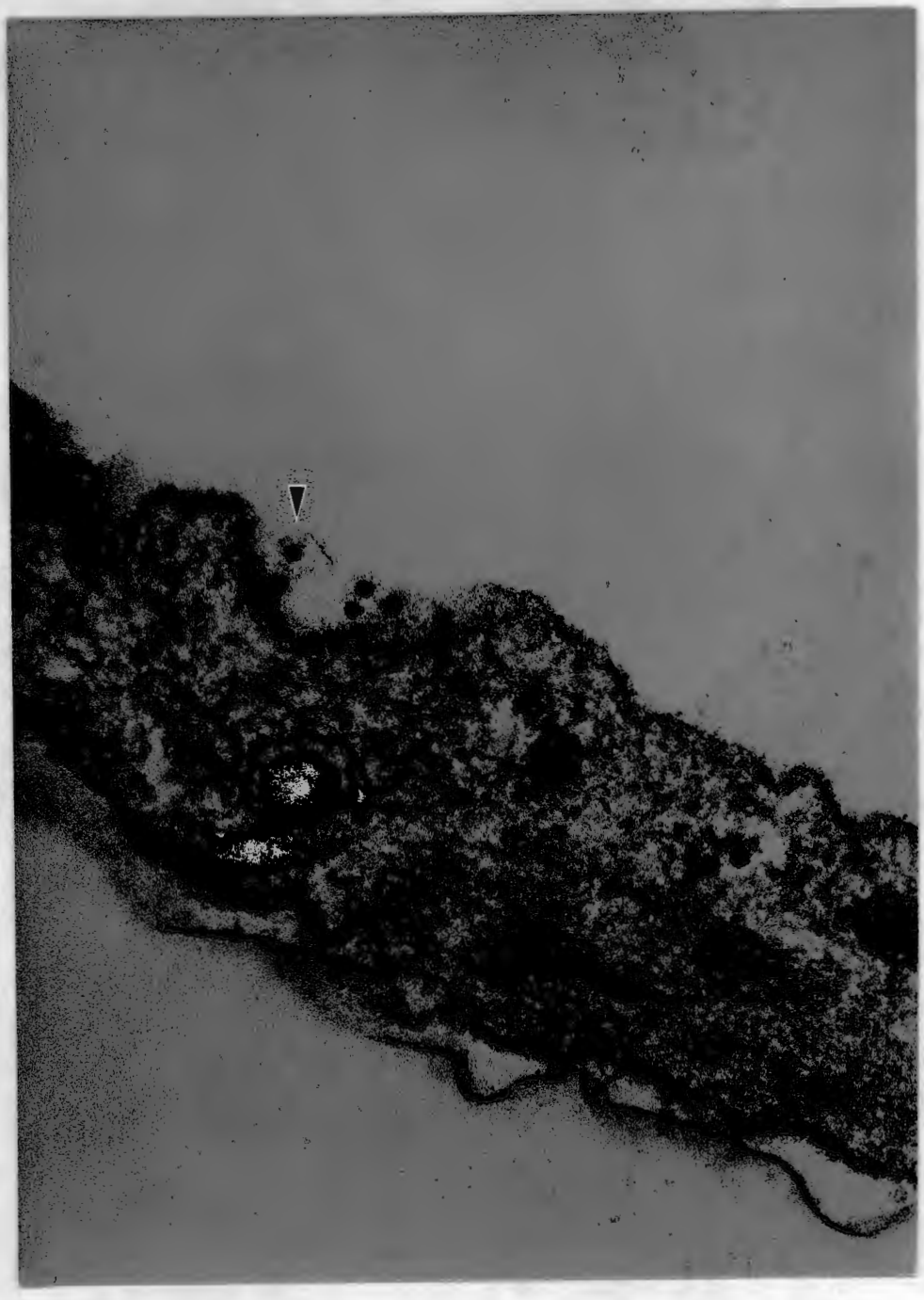


PLATE 4.11 C: Ultrathin section of subconfluent E8 cell incubated with Au-LDL at 4°C before processing for EM. One Au-LDL probe in the coated pit shown in this micrograph reveals a faint outline which may represent the LDL shell surrounding the gold core (arrowhead). It does not appear to be bound directly to the membrane possibly owing to the oblique section or to aggregation with the particle beneath it. Of the six particles in the pit, four are clearly bound to the membrane (small arrows). Magnification = 120 000x.

Plate 4.12 A

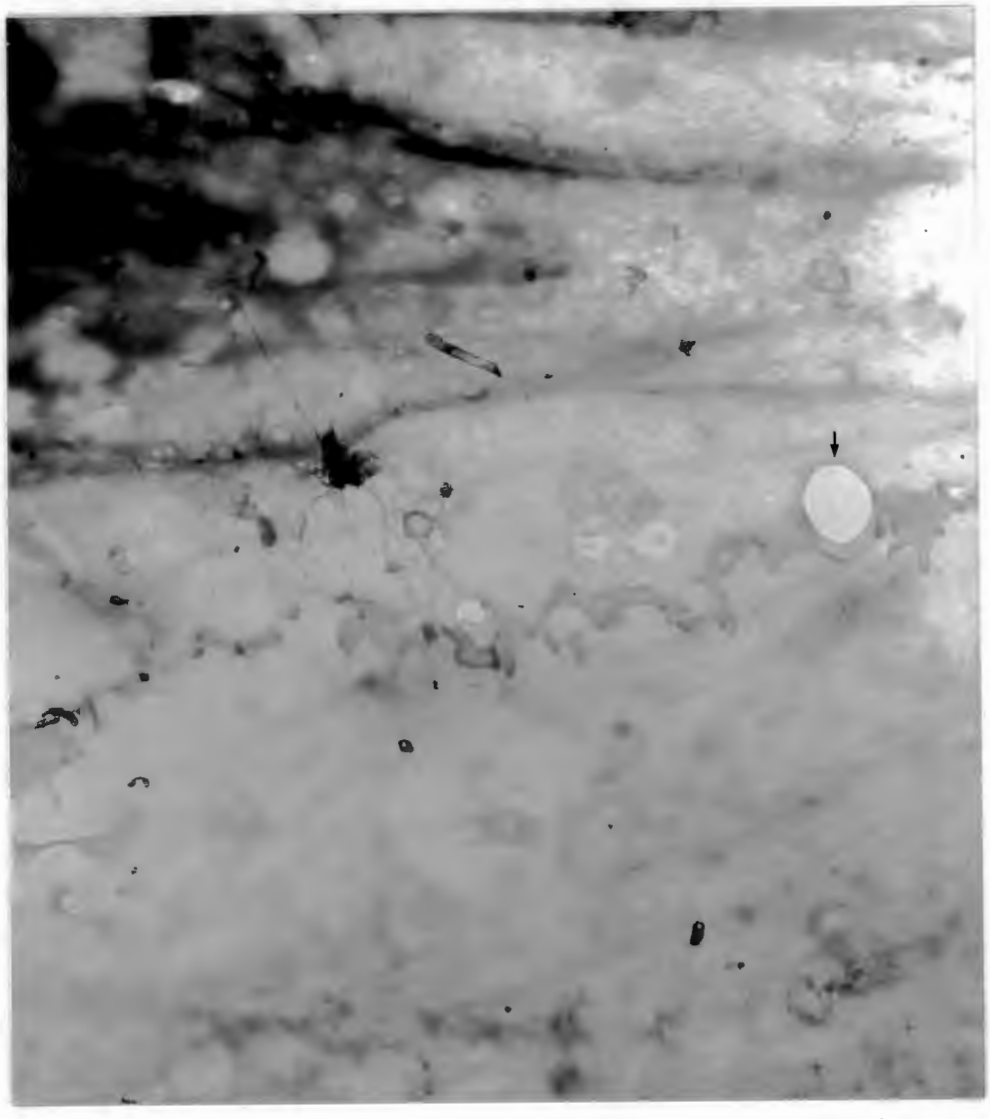


PLATE 4.12 A: Whole-mounted postconfluent monolayer of E8 endothelial cells incubated with Au-LDL at 4°C. This low magnification micrograph shows expanses of peripheral plasma membrane which are devoid of Au-LDL probes, indicating the absence of LDL-Rs on the surface of contact-inhibited E8 cells.

The junction of two adjacent cells is imaged showing interlocking overlaps at the cell margins. It is interesting to compare this image with an SEM image such as PLATE 2.9 B (Magnfication 3 700x, Chapter 2). Both images reveal scalloped cell margins but the whole-mount transmission image shows details of the overlaps which were inaccessible by scanning. Such plan views would also be difficult to interpret by transmission electron microscopy of thin-sections. The whole-mount image shows details of the overlapping and the overlapped cell margins from which the impression of an interlacing of marginal processes of the abutting cells is gained; rather like the interlacing of the fingers of hands. This complex junction probably confers great strength on the intercellular connections. The following plate shows the overlaps at greater magnification. A relatively large hole is also seen which may be an artefact caused by the shrinkage during critical point drying of the cells. Magnification = 9800x.

Plate 4.12 B

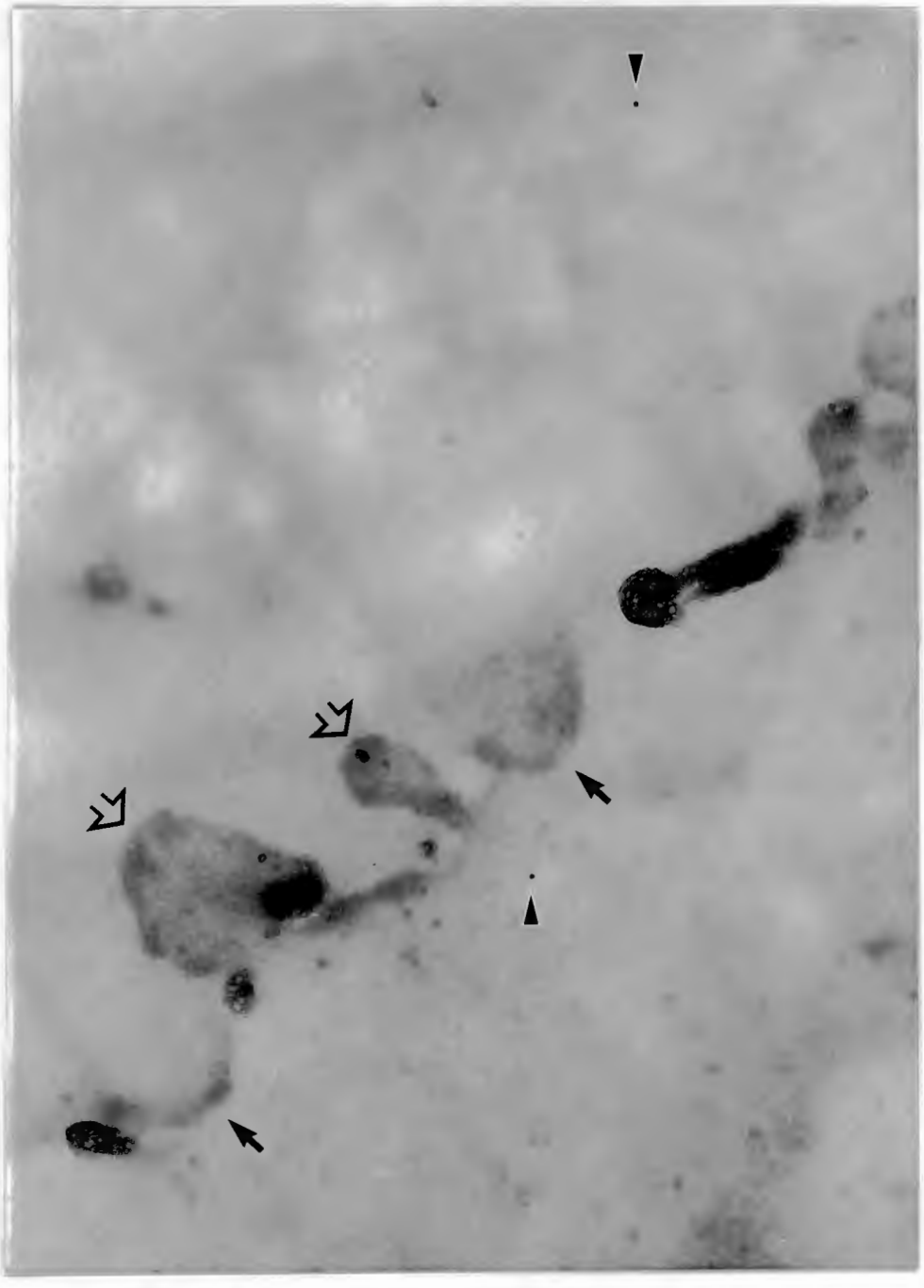


PLATE 4.12 B: Postconfluent monolayer of E8 ECs incubated with Au-LDL at 4°C. Higher magnification (37 000x) micrograph of an area in which two Au-LDL probes were located (arrowsheads). To find such areas, it was necessary to scan large expanses of contact-inhibited culture surface. The level of labelling is so low as to be considered negligible.

The interlocking overlaps of the abutting cell margins can be very clearly interpretted from this micrograph. The open arrows indicate overlapping marginal processes from the cell on the right: the solid arrows indicate overlapping processes from the left-hand cell. There is a tendency for the processes to alternate along the length of the margin (cf. PLATE 4.12 A).

Plate 4.13

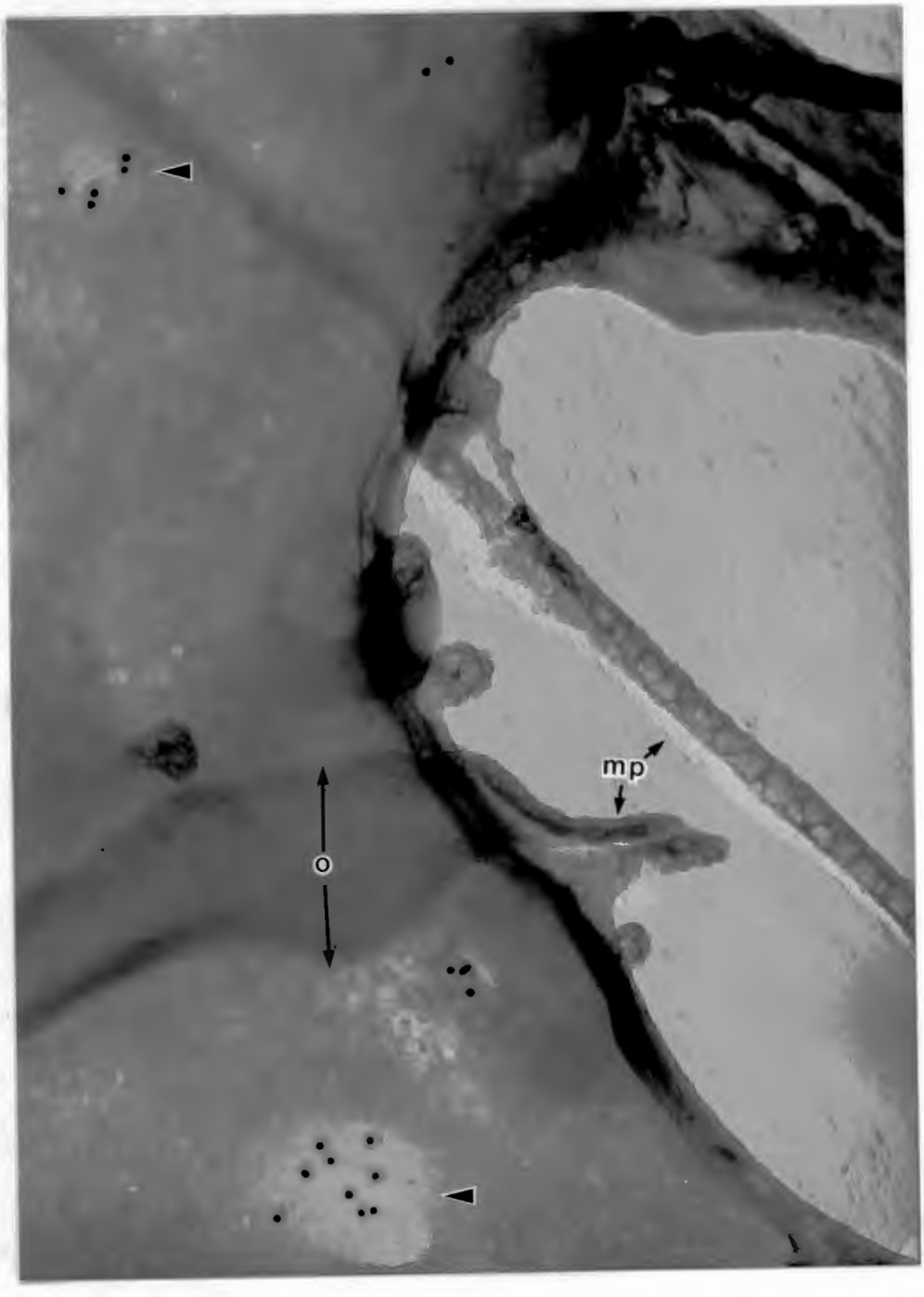


PLATE 4.13: Postconfluent monolayers of E8 cells were wounded by making multiple streaks across the dish surface with a rounded pasteur pipette tip which denuded narrow areas of cells. These cultures were allowed to regenerate for 15 hours in MEM-LPDS, exposed to Au-LDL and prepared for whole-mount transmission electron microscopy. A regenerating cell bearing clustered Au-LDL probes in clearly discernable pits (arrowheads) in the plasma membrane surface is seen here. The cell has well-developed microprocesses (mp) as well as free margins, which are consistent with spreading (motility). Note the overlap (o) indicating previously confluent cells which are probably at the edge of the wound (open space on the right). Magnification = 60 000x.

DISCUSSION

Human skin fibroblasts have been used for more than 10 years as a model system in which to study RME of LDL.[3, 101, 4] In view of the wealth of detailed, consistent morphological and biochemical data pertaining to the interaction of LDL with its receptor in fibroblasts, the ultrastructural probe techniques destined for use with ECs in this thesis, were first tested and standardised on fibroblasts.

LDL Interactions with Fibroblasts

Before discussing the surface distribution of LDL on normal and mutant fibroblasts as visualised using Au-LDL in this study, I shall briefly review the background knowledge.

Despite the disadvantages of lengthy preparation and non-specific adsorption to the cell membrane, Anderson et al.[6, 5, 100] described the binding of ferritin-conjugated LDL (F-LDL) to coated and non-coated parts of the plasma membrane. The specific, receptor-mediated nature of the binding of F-LDL was clear because FH homozygotes bound little; heparin dramatically reduced binding to coated regions in normal cells; and excess LDL had the same effect.[7] Using cell profiles from ultrathin cross-sections, Anderson et al.[7] counted ferritin cores per unit length of membrane and established that about 20 - 50% of specifically bound LDL was not in coated pits on normal cells. The remaining 80-50% was bound to coated regions which constituted only 2% of the analysed membrane length.[7] The F-LDL appeared to be marking receptors which were clustered into coated pit areas, and also others which were not. Ligand-induced clustering of LDL-Rs was excluded by fixing the cells with paraformaldehyde prior to ligand binding. Fixation influenced neither the pattern nor the efficiency of binding.

The high degree of variation in the binding percentages may have been due to sampling errors inherent in the methodology. As discussed in the introduction to this chapter, impressions of the topographical distribution of markers on cells can be gained by analysing thin sections. The assumption is that linear distribution data from a minimum number of sections (which reveal surfaces as profiles or lines) will indicate what the topographical distribution looks like. This assumption is only valid for uniformly-distributed markers analysed by a statistically significant sample of sections.[72] There is evidence for considerable variability in the binding of LDL from region to region on the surface of a given fibroblast, and from cell to cell in a given culture.[219, 217] An additional source of variation may have been contributed by the range of diameters which exist amongst coated pit microdomains at any given time. Robenek et al.[215] have described various stages of progressive clustering of receptors in coated pits, ranging from loose associations to closely-packed aggregations on the point of endocytosis.

In other studies, Anderson et al.[5] found that more than 30% of the F-LDL was bound to non-coated regions of the plasma membrane - about 98% of the analysed membrane length. The LDL bound to non-coated regions "was found scattered along the 98% of cell surface that was non-coated".[5] This implied that LDL was binding to randomly dispersed receptors. Although diffuse, it was thought to be specific binding to receptors because fibroblasts from FH homozygotes bound much lower levels of F-LDL.[7]

A pulse-chase experiment monitoring the disappearance of F-LDL from coated regions with warming at 37°C, showed that as F-LDL disappeared from coated regions, it also left non-coated regions. They suggested that, as a result of endocytosis of F-LDL in coated pits, recruitment of fresh F-LDL into coated regions from non-coated regions was occurring. They assumed that F-LDL was only taken up via coated pits because they never saw uptake via non-coated vesicles. They did not rule out the possibility that F-LDL bound to non-coated regions may have become dissociated during processing for electron microscopy. By light microscopical immunocytochemistry, clathrin positions beneath the plasma membrane of the

coated pit were seen to be in register with F-LDL foci above.[2] These co-localised antigens were arranged in linear arrays which corresponded with the positions of stress fibres within the fibroblasts. Anderson et al.[6] raised the important question of the patching of membrane antigens by monospecific antibodies, implying that clathrin or LDL-Rs might have been aggregated by the immunocytochemical methods. This point was controlled first by applying F-LDL to the receptors at 4^0C - a temperature considered low enough to prevent movement of proteins in the plane of the membrane - and then by fixation of the F-LDL /cell system before application of antibodies. The question of movement of membrane proteins at low temperature is addressed below (p. 163).

The itinerary of the LDL-R during the endocytic cycle was further elucidated as a result of the discovery of an interesting mutant fibroblast which could not internalise LDL bound to receptors on its surface. These cells (GM 2408A) were obtained from a 14 year-old (J.D.) suffering from an unusual form of homozygous familial hypercholesterolaemia. LDL bound to J.D's LDL-Rs in normal amounts, and with the same affinity as to normal cells.[7, 3] But failure to internalise LDL left it distributed over noncoated regions of the plasmalemma seen in profile in the sections. The F-LDL probe appeared as small groups, each of 2-3 particles. This may have represented microclustering of receptors or pre-aggregation of the probe itself. Only 3% of the surface-bound F-LDL was found over coated regions which accounted for approximately the same percentage of the plasma membrane area.[7] These statistics would seem to suggest a diffuse distribution. In pulse-chase experiments, after a 10 minute chase at 37^0C, only 5% of F-LDL bound to the surfaces of J.D's cells had been internalised, in contrast to 75% in normal cells.

LDL bound to the surface of J.D's cells appeared as punctate foci when seen by indirect immuofluorescence techniques. These foci were smaller and more numerous than those seen in normal cells.[8] Anderson et al.[8] found by electron microscopy of J.D's cells that LDL-ferritin probes bound to non-coated regions in groups of two or three over 50 nm lengths of the plasma membrane while in normal cells larger groups were seen over 100 nm lengths.

Anderson[3] speculated that LDL-Rs might be inserted into the membrane of fibroblasts in groups of four. He based this speculation upon the following observations. Normal fibroblasts will bind four times as many LDL particles as cholesterol-rich high density lipoprotein, HDLc. HDLc, which is similar in size to LDL, has a 23-fold higher affinity for the LDL-R than LDL. The LDL-R has two ligands: apolipoproteins B and E. HDLc is rich in apolipoprotein E, which has a higher affinity for the LDL-R than apolipoprotein B (found in LDL). Anderson therefore speculated that this binding of four times as many LDL particles than HDLc might be explained by four receptors binding one HDLc particle, and one receptor binding one LDL particle. However in the light of recent structural analyses of the LDL receptor molecule, this cannot be true since there are four Apo B binding sites per receptor.[101] It is also true that Apo B and Apo E share a common binding domain on the receptor; the affinity of this receptor is higher for Apo E than for Apo B. Thus four LDL particles or a single HDLc particle binding to a receptor molecule could also account for the data. Whatever the fine points of receptor-ligand stoichiometry, the degree of clustering of LDL-R in J.D's cells has not until now been probed topographically in an ultrastructural study. Such a study using a receptor probe, Au-LDL, in conjunction with whole-mounted GM 2408A cells has been reported in this chapter. The LDL-Rs probed with Au-LDL, were not organised into clusters, but were monodisperse and randomly distributed over regions of the cell surface. Occasionally, Au-LDL probes were seen in groupings of two, three or rarely four. These groupings may have been caused by a low level of aggregation of the probe molecules themselves and may not reflect receptor aggregation at all (PLATE 4.2 A).

Although at a lower efficiency £ , a similar monodisperse distribution LDL-R was detected on J.D's cells by the antireceptor monoclonal antibody IgG-C7[17] used in this work. This alternative receptor probe was used in conjunction with immunogold techniques. Only single gold labelled probes rather than clusters were detected randomly distributed over the surfaces.

Considerable support was given to these findings by the LDL-R distribution on normal fibroblasts (FGo)and receptor-negative mutants (GM 2000) detected by the same methods. Au-LDL binding to receptor-negative cells was negligible. Normal cells (FGo) had two clear populations of receptors: (i) diffusely-distributed singletons and occasional small groups of 2-4 and (ii) highly organised clusters, usually comprising ten or more receptors; a picture entirely consistent with RME via coated pits. These clusters varied in diameter from 200 to 500 nm and were frequently delineated by a ring of Au-LDL probes. Again, these results were confirmed using the C7 monoclonal antibody probe. The absence of a dispersed population of Au-LDL probes on receptor negative cells shows that the dispersed probe distribution on normal and GM 2408A cells was not caused by non-specific binding. The level of non-specific binding was so low on GM 2000 as to be negligible. This feature of the Au-LDL probe made it preferable to the C7 system which like most indirect immunocytochemical procedures has problems of non-specific binding of the secondary antibody.

The almost monodisperse distribution of receptors on J.D's cells as shown with Au-LDL has important implications regarding the deployment of receptors on the fibroblast surface before their organisation into coated pits. It suggests that newly synthesized receptors are inserted singly and that clustering takes place after this. Basu et al.[16] found that recycling of LDL-R is unlikely in J.D's cells because their treatment with monensin failed to trap receptors in internal pools. Thus, in this study the dispersed population of Au-LDL probes may have localised newly-synthesized receptors which have been inserted into the plasma membrane.

In order to determine the dynamics of newly-inserted receptors, time-course studies need to be done. However, it could be argued that at any instant, some receptors will just have arrived at the surface and if clustered, will be retained in this pattern unless the rate of diffusion is so fast that the existence of such groupings is transient. Clustered receptors were not detected on J.D's cells in such a time-sample experiment, so it is likely that either the newly-synthesized receptors were inserted singly or that, if preclustered, they diffused rapidly to assume a monodisperse pattern.

Considering the monodisperse population co-existing with clustered receptors on normal fibroblasts in the light of the findings on J.D's cells, it is possible that even recycled receptors are either inserted singly or diffuse rapidly to produce a monodisperse population which is later recruited into clusters .

Robenek et al.[215] found no evidence of diffusely distributed receptors outside of what they termed "plaques" on fibroblasts. They argued for the existence of surface receptors organised into large clusters which became associated with coated pits. The findings reported are in contrast with those of Robenek inasmuch as a monodisperse population of receptors was found to co-exist with clustered receptors.

£ The monoclonal antibody C7 probed with a colloidal gold-labelled goat antimouse IgG (GAM-G15) secondary antibody was relatively inefficient at detecting LDL receptors. The inefficiency probably resided in the interaction between GAM-G15 and C7, not between C7 and the receptor. The efficiency with which colloidal gold-labelled antibodies bind to their antigens appears to be inversely related to the size of the gold particles (C.R. Hopkins, personal communication). The IgG molecules appear bound to the surface of the gold particles as a thin shell. It is possible therefore that some binding sites might be hidden as a result of electrostatic binding to the gold. This would adversely affect the efficiency of binding of the probe since fewer sites would be available. In addition, the size of the gold particles probably constitutes steric hindrance to the efficient binding of adsorbed antibody with antigen.

LDL Interaction with Endothelial Cells

Using C7 monoclonal antibodies and Au-LDL as ultrastructural probes for the LDL-R on the surface of active endothelial cells, the coexistence of two populations of receptors has been demonstrated here, as in normal human skin fibroblasts, The first is a largely monodisperse population located at random over the peripheral cytoplasm of the spread cells. The second population is composed of clustered receptors organised into ring-shaped arrays situated on the plasma-membrane above coated regions or frank coated pits. The LDL portions of the probes were sometimes faintly visible (PLATES 4.11 A,B,C) and these touched the plasma membrane in most cases. The distance between the gold core of the Au-LDL probe and the plasma membrane to which it was apposed was approximately 20 nm. This further supported the case for receptor-mediated binding to the membrane above coated regions. It is tempting to speculate that the dispersed population represented the insertion of newly appearing recycled receptors when it was seen at the extreme margins of the cell peripheral cytoplasm.[31] Indirect evidence for this was perhaps provided by the fact that clustered receptors were never seen as close to the cell margins as monodisperse receptors.

Receptor Itinerary

A model whereby recycled receptors insert singly or in small groups which later become organised into coated pits is consistent with the ideas of Anderson et al.[3] and inconsistent with those of Robenek et al.[215] who argue for preclustered recycling of LDL-Rs in fibroblasts. The latter hypothesis must postulate the organisation of coated pits beneath these clusters by some unknown mechanism. It is not profitable to discuss the separate existence of these clusters from coated pits, nor to speculate that recruitment of single receptors by coated pits might occur. To address such issues it will be necessary to perform time-course experiments and to probe for LDL-R and clathrin in order to localise receptors and coated regions simultaneously.

The recycling itinerary of a different receptor, the transferrin receptor, has been elegantly analysed by Hopkins[138, 136] in human epidermoid carcinoma A431 cells. These cells can express up to 400 000 receptors per cell (Hopkins, personal communication) which can be localised by a monoclonal antibody (B3/25).[136] Colloidal gold particles conjugated with B3/25 to produce an electron-dense anti-transferrin receptor probe were used to map newly-appearing transferrin receptors on the surfaces of A431 cells in time-course experiments. By saturating exposed receptors with unlabelled B3/25 monoclonal antibodies, and then probing for newly-inserted receptors with gold-labelled B3/25 after various time intervals, Hopkins was able to map their dynamics. Receptors were initially monodisperse, then they associated progressively forming packed arrays in coated microdomains (pits). Recycled receptors inserted at the leading edges of A431 cells and moved centripetally during their progressive association into clusters over coated pits.[136]

These dynamics agree with the ideas of Bretscher[31] that recycling receptors insert together with recycling membrane at the leading edges of motile cells. Membrane additions carrying receptors with them create a membrane flow away from the leading edge on both upper and lower cell surfaces. Bretscher[30] demonstrated marked preferential distribution of both LDL and transferrin receptors at the edges of spreading HeLa cells. The marginal insertion and centripetal flow of transferrin receptors in A431 cells shown by Hopkins[136] would appear to support these ideas.

Although no time-course experiments were conducted to probe the LDL-R itinerary in this study, the monodisperse population of receptors did appear to be located at the extreme periphery of subconfluent ECs which were actively spreading (see above). This was not so noticeable in the fibroblasts used here as they were preconfluent and not so active.

Movement of Receptors at Low Temperature

The binding of LDL to fibroblast receptors at 4°C does not cause ligand-induced clustering as shown by prefixation studies.[6] Therefore, in fibroblasts this temperature is low enough to prevent the movement of receptors in the plane of the membrane. Hopkins (personal communication) advocates fixation of systems before applying immunocytochemical and direct probes. While this might result in some denaturation of receptors, with concomitant loss of affinity for antibodies or ligands, it may be prudent since some membrane proteins are said to move at low temperature. An example is given by the different behaviour of two receptors. α2-macroglobulin receptors like those for EGF, are monodisperse on cell surfaces. In the presence of ligand or divalent antibodies, EGF receptors remain immobile at 4°C but α2-macroglobulin receptors are mobile and can be aggregated.[282]

Temperature-dependence of receptor movement may have some bearing on the discrepancy between the work reported in this chapter and that of Robenek et al.[219] who did not detect a disperse population of receptors on normal fibroblasts. If, after having bound the Au-LDL to the cell surfaces, the temperature of cells and fluids was not kept low enough, receptors may have continued to move and to aggregate during the washing steps before immobilisation by fixation. In this way a monodisperse population would go unnoticed. A second possibility is that the Au-LDL probes used by Robenek were aggregated even before application to the cells. The manner in which Au-LDL probes were processed after conjugation could account for aggregation . In the study reported here, probes were washed in albumin-containing buffer in order to remove the free LDL and sucrose from the first centrifugation. Probes were then concentrated by further centrifugation. When checked by electron microscopy immediately after these centrifugations, probes were mainly monodisperse with very occasional small aggregates of two, three or rarely four probes. In a different method, that used by Robenek et al.[219] in their early work, probes were dialyzed overnight to remove sucrose from the first centrifugation step. When probes treated in that manner were examined in the EM, they were found to be aggregated and contaminated with free LDL. It is interesting to note that published micrographs of Au-LDL probes contain free LDL[189, 190, 191] and aggregated probes.[216] If experiments were indeed performed with pre-aggregated probes then monodisperse receptors would be labelled with aggregates of various sizes and would appear to be clustered.

Topographical Ultrastructure

Not all cells in normal fibroblast or endothelial cultures were equally endowed with surface receptors for LDL - often one cell might be heavily marked with Au-LDL probe while its neighbour had none. This extreme case was very clearly illustrated by the wounding experiments in ECs and can be explained by the state of quiescence of most of the cells. Regenerating cells in the wound, requiring cholesterol, expressed receptors for LDL. In fibroblasts inter-cell variability of LDL-R expression was not so dramatic as these cells did not reach total quiescence during density-dependent contact-inhibition.[150] Thus the occurence of cells totally devoid of receptors was rare, unlike the situation in postconfluent ECs.

Rings of Au-LDL probes marking annular receptor distribution in surface depressions on fibroblasts and endothelial cells were described in this chapter. This phenomenon was confirmed with C7 monoclonal anti-receptor probes used in conjunction with gold-labelled antibodies. The large size of the Au-LDL probe could conceivably have restricted its binding to the periphery of a coated pit filled with receptors; more central binding may have been prevented by steric hindrance. But the C7 probe, much smaller than Au-LDL, would have comparatively unrestricted access to most receptors, whether placed in central or peripheral regions of a coated pit. Additional support for this ringlike distribution of receptors round the coated pit was found in the work of others. Preferential binding of F-LDL on the edges of coated regions. of sectioned fibroblast plasma membranes was noted by Anderson et al.[6] Heuser[134, 3]

imaged native LDL bound to coated pit regions on normal and mutant fibroblasts using his elegant rapid-freeze, deep-etch techniques. Although the feature was not discussed, his micrographs revealed a ring-shaped arrangement of LDL on a normal cell surrounding a coated pit. Perhaps these rings of receptors are formed by closure of linear arrangements of receptors which form during the association of monodisperse receptors?

The plasma membrane areas enclosed by rings of Au-LDL probes were positioned above coated areas, or incipient coated pits. In several images these areas appear to be smoother than the surrounding, presumably non-coated, regions (PLATES 4.6 D; 4.9 C,D; 4.10 A,B). Similar smooth microdomains have been reported by Hopkins[136] who probed for transferrin receptors with a gold-labelled anti-transferrin antibody. Although in the present study it is possible that these smoother areas were protected from shadowing because they were low-lying, higher magnification micrographs (PLATE 4.10 A,B) reveal a surface of fine texture enclosed by Au-LDL probes. These gold-delineated microdomains may be adjoined by others not marked at all (PLATE 4.10 A) since not all coated pits necessarily contain LDL-R.[6]

APPENDIX TO CHAPTER FOUR

Indirect Immunogold Staining and Whole-Mount Methods

IMMUNOGOLD LABELLING

PROTOCOL

Fibroblasts were cultured in plastic dishes until they were preconfluent, having many flattened cells with thinly spread peripheral cytoplasm. To detect, at the ultrastructural level, LDL receptors exposed on the cell surfaces, the following protocol £ was carried out on cells in culture dishes:

1. Cells washed briefly, twice in MgPBS

2. Cells chilled to $4^{o}C$ for 10 minutes

3. IgG-C7 monoclonal antibody incubation for 60 minutes at $4^{o}C$
   Diluent: 0.5% BSA in MgPBS, pH 7.4

4. Four washes in MgPBS containing 0.2% BSA at $4^{o}C$

5. Three washes in MgPBS at $4^{o}C$

6. Fixation in 3% paraformaldehyde in MgPBS, pH 7.4
   for 10 minutes at $4^{o}C$ and 10 minutes at $20\text{-}25^{o}C$
   (From this point on, the procedural steps were at $20\text{-}25^{o}C$).

7. Two washes in MgPBS, 5 minutes each

8. Blocking with 3% BSA in MgPBS for 30 minutes

9. Brief wash in tris-HCl buffer, ph 8.2, containing 1% BSA

10. Incubation with GAM-G15, diluted 1:20 in tris-HCl, pH 8.2,
    for 60 minutes.
    The gold probes were centrifuged at 8000 xg for 2 minutes
    before use to remove aggregates.

11. Two washes in tris-HCl buffer, ph 7.4, containing 0.2% BSA

12. Two washes in tris-HCl buffer, ph 7.4

13. Final fixation in 2.5% glutaraldehyde (EM grade, Merck,
    Darmstadt, West Germany) in MgPBS.

14. Whole-mount processing as detailed below.

£ Steps 1 - 6 were based on the methods of Anderson reported in Beisiegel et al.[17]
Steps 7 - 14 were based on methods described by Hopkins,[136 - 138] Tolson et al.[275] and De Mey.[68]

WHOLE-MOUNT TRANSMISSION ELECTRON MICROSCOPY OF CELLS CULTURED ON GLASS AND STYRENE PLASTIC SUBSTRATA.

INTRODUCTION

Multistep immunocytochemical procedures for labelling intra- or extracellular antigens in cultured cells usually involve repeated exchanges of liquids over prolonged periods of time. Traditionally, cells for examination whole in the transmission electron microscope have been prepared from glass coverslip cultures.[41] These were fixed, critical-point or air-dried, coated with platinum and carbon and released from the glass surface with the aid of dilute hydrofluoric acid.[275] The coated cells were collected directly onto grids from the surface of a water trough. In the course of the present investigation, it was found that human skin fibroblasts and bovine aortic ECs detached and were lost progressively from glass coverslip surfaces throughout prolonged immunocytochemical processing. The unavoidable turbulence accompanying fluid exchanges and the duration of the procedures caused this detachment and cell loss. Cell adhesion to plastic was observed to be firmer than to glass substrata. Therefore a method was developed for releasing dried, coated cells from the styrene dish surfaces and using these cells as whole-mounts for transmission electron microscopy. The method employed propylene oxide to release the coated cells by limited dissolution of the styrene substratum.[161]

MATERIALS and METHODS

Cells on Plastic Substrata

Fibroblasts and ECs were cultured in regular styrene tissue culture dishes or special dishes with four circular internal wells 1-2 mm deep and 8 mm in diameter (Greiner 627 170), suitable for individual immunocytochemical tests. Unless otherwise indicated, the following procedures were conducted at room temperature (20-25^{o}C). After completion of an immunocytochemical procedure, cells were fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate (pH 7.2) buffer, washed twice in buffer, osmicated in 1% OsO^4 in buffer for 30 minutes and rinsed in distilled water. Dehydration through a graded series of aqueous ethanols (35, 50, 70, 96%, 10 minutes in each different solution) preceded three soaks, each 10 minutes long, in absolute ethanol (chemically dried with anhydrous sodium sulphate). The dishes were critical point dried using absolute ethanol as intermediate fluid and carbon dioxide as transitional fluid. Three cycles each comprising a 2 minute purge and a 10 minute soak in CO_2 preceded the final soak and critical temperature steps described in Chapter 2. If the dishes were exposed to liquid CO_2 for longer than 60 minutes, the styrene softened at temperatures 2 - 3 degrees above critical (31.1^{o}C). Apart from this problem, bubbles of gas appeared in the plastic during the heating and pressure reduction cycles. Blank dishes were tested in a dummy cycle to determine the maximum carbon dioxide exposure time.

The dried material was immediately tranferred to a vacuum desiccator or directly into a Balzers BAE 121 coating plant which was pumped down to a vacuum better than 5 x10^{-6} Torr. The shadowing was achieved by electron beam evaporation of a platinum/carbon target using a Balzers Electron Beam Gun EK552 at 1700V, 70mA at a distance of 10 cm from the cells. The specimen rotary-shadowed at 35^{o} to the beam for 5-10 seconds and normal to the beam for a further 5-10 seconds. The platinum/carbon shadowed specimen was then coated with a carbon layer 10 -20 nm thick evaporated from carbon electrodes at 16V, 8A.

Using a needle, areas of interest on the coated surface of the plastic dish were scribed off into tiny squares of side about 2 mm to cover nicely the viewing area of 3 mm grids. Individual

wells were cut out as discs from the underside of the dishes using a brass hole saw in a drill press. The cell side was completely undamaged by this cutting procedure. Discs were dipped into propylene oxide (1,2-epoxy propane) which released the squares of carbon/platinum film . The thin films of cells were collected directly on grids (400 mesh copper) dipped into the propylene oxide beaker in which the squares were swirling about. It was important to leave the films in the solvent for 2 - 3 minutes to allow sufficient time for the plastic to be completely dissolved from the films.

Cells Cultured on Glass Coverslips

Cells on glass coverslips were processed identically to cells on plastic up to the critical point drying step. Removing the scored, coated cell layers from glass surfaces was achieved by dipping them slowly into 5% hydrofluoric acid at an angle in order to float off the coating squares. The coverslip , when completely submerged and free of squares was allowed to sink to the bottom of the trough. Copper grids, held between jeweller's forceps were used to pick up the floating squares. Preparations were washed in distilled water and laid on filter paper to dry.

Air-drying

In cases not requiring a high degree of preservation of surface detail, the critical point drying was replaced by air-drying immediately after the last absolute ethanol soak. Still wet, dishes were placed in a dry incubator at $40^{o}C$ for 1-2 hours, then coated as described above.

Electron Microscopy

Once collected on grids, whole-mounted cells were viewed in the transmission electron microscope at 80-100 kV. For thicker parts of specimens, voltages of 120 kV were sometimes used.

RESULTS and DISCUSSION

PLATES 4.3 - 4.10, 4.12 and 4.13 show human skin fibroblast and EC whole-mounts prepared by the techniques described here. The cell surfaces have been marked with different colloidal gold probes for the LDL receptor. There is a difference in quality between fibroblasts (PLATES 4.3 - 4.8) and ECs (4.9, 4.10, 4.12 and 4.13) prepared by identical methods. In general the fibroblasts were severely damaged (possibly by the immunocytochemical procedures) and appeared to have lost membrane material. FGo cells (normal fibroblasts) were less susceptible to membrane loss than the mutants GM 2000 and GM 2408A. In contrast ECs were well-preserved and had smooth plasma membranes. Surface ultrastructure like microvilli, coated pits and marginal processes were well-preserved.

The advantages of preparing whole-mounts from cells grown on plastic substrata are summarized below.

1: Cell losses during processing were minimized because of strong adhesion of cells to the styrene substratum.

2: It was more convenient to use pre-sterilized (or pre-coated) plastic labware than glass coverslips which needing sterilisation and individual positioning in dishes. The washing of coverslips during processing presents special problems as does the handling of these delicate objects. Plastic dishes were easily handled and cells could be processed directly in situ.

3: Postconfluent endothelial cell morphology depended upon the substratum. Contact-inhibited monolayers of postconfluent bovine aortic ECs formed more readily on styrene plastic than on glass (unpublished findings).

Where large numbers of specimens required processing (as in the present study), the use of plastic culture dishes as culture substrata considerably improved the efficiency of the microtechniques when compared to the use of glass coverslips. The techniques described above were specially developed in the course of this study of the expression of LDL receptors on the surface of cultured cells.

CHAPTER FIVE

Concluding Discussion

LDL Interactions with Endothelial Cells

In this thesis, I have concentrated on the receptor-mediated endocytosis of LDL by cultured bovine aortic ECs in order to learn something of the mechanism whereby ECs control LDL receptor activity as they modulate from active growth to contact-inhibition. It has been seen in Chapters 3 and 4 that a progressive decline of LDL receptor activity correlated with the modulation of cells from a proliferative phase to a contact-inhibited quiescent phase. The active cells were of distinctly different morphology from the contact-inhibited cells. This morphological transformation to postconfluent monolayering (see Chapters 2 and 3) correlated strongly with the minimal expression of LDL receptors. LDL surface-binding was vanishingly low in postconfluent cells as detected by ^{125}I-LDL binding, fluorescence microscopy of DiI-LDL, and Au-LDL and C7 antireceptor monoclonal antibody probing at the ultrastructural level. As expected, the endocytosis and degradation of LDL decreased dramatically in postconfluent ECs as reported in Chapter 3.

The progressive decline of LDL receptor activity was also correlated with a progressive increase in AcLDL receptor activity. This was measured by binding, endocytosis and degradation of ^{125}I-AcLDL, which showed proportionate increases in cultures maturing to contact-inhibited monlayers. DiI-labelled AcLDL confirmed the radiochemical data and provided distribution data which has been discussed in Chapter 3.

Postconfluent ECs differ from other cell types such as SMCs and fibroblasts by their strict contact-inhibition of proliferation and their refractory response to LDL. Unlike ECs, SMCs and fibroblasts do not form contact-inhibited monolayers at confluence, but continue to proliferate albeit with decreasing vigour, eventually forming multilayered cultures. Presumably, in vivo there are contact-inhibition mechanisms (triggered by three-dimensional growth) which limit proliferation. In contrast, ECs appear to have controls triggered by two-dimensional space-filling as the cells form monolayered sheets. The modulation of LDL metabolism mirrors growth behaviour. SMCs[20] and fibroblasts[160, 150] also have depressed LDL receptor activity at confluence, but the extent to which it is depressed is less dramatic than that of ECs.[271, 151]

In a similar study,[94] total LDL endocytosis and degradation rates (receptor-mediated plus receptor-independent) were reduced in the postconfluent ECs[271] similar to those used here. Van Hinsbergh et al.[279] also reported reduced uptake and degradation of LDL by pinocytotic, adsorptive and receptor-mediated endocytosis by human ECs in vitro. Davies et al.[67] reported the reduction by one-third of the rate of fluid pinocytosis in confluent bovine aortic ECs in culture from that measured in actively proliferating cells. At full contact-inhibition, receptor-independent endocytosis became predominant although much reduced overall when compared with active cells.[279] In vivo it is likely that this component would be concerned mainly with transcytotic processes[280] which are not ideally investigated in cell culture.

The results reported in this thesis indicate that it is within the potential of ECs not to express any LDL receptors on the surface if there is no need for cholesterol. This need appears to be strongly correlated with cellular growth.

The mechanism of end-product feedback inhibition of LDL receptor numbers[32] can explain the extremely low rates of LDL endocytosis and degradation in postconfluent ECs in culture (discussed fully in Chapter 3). Briefly, the present finding that LDL receptor expression was undetectably low strongly supports the above mechanism but not that of Vlodavsky et al.[77, 284, 286] Those authors suggested a mechanism of restricted lateral diffusion of expressed LDL receptors which still bind LDL to the surfaces of confluent ECs. The putative restriction of movement is thought to inhibit internalisation or endocytosis of already bound LDL - albeit reduced in quantity. Kenagy et al.[151] addressed the problem of reduced LDL metabolism: they found low binding levels in confluent cells and also reported a proportional decrease in internalisation and degradation of LDL. The growth rate and LDL receptor activity were correlated by comparison of thymidine indices with LDL metabolism. The separation of the

influences of growth rate and cell density upon the binding and metabolism of LDL proved difficult in the EC model. However, a growth-independent component which was probably related to cell density in fibroblasts was reported by the same group.[150]

The most important finding of the present study was the almost total absence of detectable LDL receptors on the surfaces of contact-inhibited monolayers of aortic ECs. Radioactive, fluorescent and ultrastructural probe techniques, three techniques of increasing sensitivity were used to establish this. The lower limit of the assay of bound ^{125}I-LDL has already been discussed in Chapter 3 (p.112) where it was suggested that bound LDL levels on postconfluent cells were likely to be lower than those measured. Barak and Webb[14] have calculated that each particle of DiI-LDL (suitably labelled at high enough specific activity) carries approximately 45 DiI molecules, and that this is sufficient to render individual LDL particles detectable in the fluorescence microscope. The practical limit of detection of bound DiI-LDL was essentially the photosensitivity of the detector (in this case the eye). Since no image intensification apparatus was available, the absence of DiI fluorescence on postconfluent cells incubated with DiI-LDL could not be taken as absolute proof of lack of LDL binding, and therefore absence of receptors. To solve this problem, ultrastructural probes were used which had the added advantage of giving ultrastructural information.

The Au-LDL ultrastructural probe, once bound, has no lower limit of detectability like radioactive and fluorescent probes: even a single Au-LDL probe can be detected in the electron microscope after thorough searching. In this study the virtual absence of background or non-specific binding gave confidence that bound Au-LDL was indeed probing receptors. Extremely low binding to receptor-negative fibroblast controls and heavy binding to receptor-positive fibroblast controls established this point. The almost total absence of probes on postconfluent ECs indicated strongly the corresponding lack of receptors. In further support of these findings was the heavy labelling of subconfluent ECs and regenerating ECs in wound areas of previously contact-inhibited cultures (see Chapter 4).

When considering individual cells culture, it is clear that ECs have the potential to be totally quiescent with respect to LDL receptor-mediated binding and endocytosis. However, extrapolation of these conclusions to predict a correspondingly low level of LDL receptor on the endothelium of the normal aorta of man or other animals would be premature. The intimal surface may indeed prove to have silent areas which have no LDL receptor-activity at all, but other regions of relatively high activity are also expected.[223] Repair or regenerative growth must be taking place continually as cells are shed owing to senescence, shearing forces, and pathological processes. LDL receptors would then be expressed by migrating and proliferating cells regenerating the endothelium in these damaged areas.

LDL Uptake by Endothelial Cells

LDL uptake can occur via two major routes in ECs:
1) receptor-mediated endocytosis (discussed in Chapters 1 and 3), and
2) receptor-independent endocytosis, which includes transcytosis.
Both processes occur simultaneously but their relative contributions to uptake depend upon the LDL concentration. At low concentrations (near the Kd of the LDL receptor, 20µg/ml) receptor-independent uptake did not exceed 20% of the total in studies carried out on bovine aortic ECs in culture.[271] But on the other hand, at high concentrations of LDL (200 - 500µg/ml) which are similar to those found in vivo, receptor-mediated uptake of LDL is proportionally much less.[279]

Receptor-Independent Endocytosis

Wiklund et al.[294] measured the relative contributions of receptor-mediated and receptor-independent endocytosis to total LDL flux into the aortic intima of rabbits. The flux

measurement was based on LDL within the intima at the times of sampling: the possibility existed that some of these LDL might later have left the intima by retro-endocytosis or reverse trancytosis. Reductively-methylated LDL (Me-LDL, which is not bound by the LDL receptor)) and native LDL (labelled with different isotopes) were used to estimate receptor-independent flux and total flux respectively. There was no significant difference between the two which indicated that receptor-mediated mechanisms played little, if any, role in the overall flux of LDL into the intima. At physiological concentrations of LDL, receptor-independent endocytosis of LDL predominates as shown in the studies by Van Hinsbergh et al.[279] in cultured human arterial ECs. In adition, morphometric studies of the transcytosis of LDL by rat aortic EC in situ by Vasile et al.[280] have shown that the endothelial vesicular pathway, not the receptor-mediated endocytic pathway, is responsible for transcytosis. These findings are consistent with the predominant role of receptor-independent processes in intimal LDL uptake when considered together with the flux measurements of Wiklund et al. Steinberg et al.[267] pointed out that the morphometric approach cannot measure flux (rate of particle flow) but only the number of particles in the pathways at the time of sampling. Wiklund et al., however, measured intimal LDL levels at 30 and 60 minute intervals and found an almost linear increase with time. Besides confirming the existence of an LDL flux, a very limited efflux from the intima (5%) over the experimental period was noted. Steinberg et al.[267] postulated the existence of efficient reverse transport mechanisms to remove the cholesterol which would otherwise accumulate to excess in the intima. Approximately 12% of the LDL flux into the rabbit intima was degraded, and of this 12%, approximately 40-50% (i.e. 5-6% of total) was degraded by receptor-mediated processes.[267] Steinberg et al.[267] considered this 5% receptor-mediated component as being below the limit of discrimination of the Me-LDL/LDL flux assay technique. This 5%, however, represented a significant proportion of the total daily LDL catabolism in the rabbit.[47]

Receptor-Mediated Endocytosis and Degradation

Carew et al.[47] measured the receptor-mediated component of LDL degradation taking place in the intima by receptor-mediated endocytic mechanisms. The relative rates of degradation of tyramine-cellobiose-labelled LDL (TC-LDL) and tyramine-cellobiose-labelled methylated LDL (TC-Me-LDL) were determined. The tyramine-cellobiose moiety cannot escape from cells after degradation of the whole particle and so serves as a cumulative index of degradation. This is the "trapped ligand" methodology whereby degradation sites can be measured and mapped effectively in specific tissues.[206] The methodology only yields a true index of site-specific degradation rates at "infinite" time, which was calculated by computer modelling to be 24-48 hours, in practice, for the rabbit intima.[46] Carew et al. assert that the LDL receptor must therefore be expressed in vivo in the aortic intimal endothelial cells, which had previously been thought to have extremely low levels of receptor on the basis of in vitro studies.[60, 286]

It may be possible to explain the apparently high rates of receptor-mediated LDL degradation in the rabbit intima in alternative ways. The intima is known[21] to contain significant numbers of smooth muscle cells which may be responsible for some LDL degradation ascribed to endothelial cells only.[47] In addition, it is conceivable that TC-LDL is more susceptible to chemical modification than TC-Me-LDL in the rabbit intima. If so, then it may have appeared as if TC-LDL was being degraded via the LDL receptors, when the scavenger receptors might have been responsible. ECs can have a high AcLDL receptor activity in confluent cultures which leads to efficent lipoprotein degradation.[265, 271] This potential may also exist in vivo.[203] as well as the ability to modify LDL.[80] As mentioned previously, there is a precedent for the existence of modified LDL within the intima of atherosclerotic arteries.[135] In situ modification of LDL may lead to degradation at those sites which could have atherogenic consequences.

General

There are organs served by fenestrated capillaries or sinusoidal vessels (liver, spleen, bone marrow, and adrenal) whose parenchymal cells have direct access to circulating lipoproteins. This has obvious physiological significance in the cases of the liver and adrenal which express the highest densities of LDL receptors and which also have the highest uptake of LDL. The significance of the above concerns the role of ECs in vivo in the regulation of plasma cholesterol levels. It would appear that the free access of the plasma lipoproteins to the liver would make it unnecessary for the endothelium to play a significant role in the degradation of LDL, as suggested by Steinberg et al.[267] However, should the liver receptors not cope with the clearing the plasma of cholesterol-rich lipoproteins (IDL) then LDL build-up occurs. If these LDL were to become modified for uptake by the scavenger receptors, then ECs (particularly in sinusoidal ECs[203]) might play an important role in degrading modified LDL. The cholesterol esters thus produced in the ECs could then be carried to the liver via HDL in reverse cholesterol transport.[187]

The interactions of low density lipoproteins with the endothelium in vivo might be summed up as follows. Proliferating ECs express LDL receptors and endocytose LDL until growth is contact-inhibited, thereby abolishing the demand for cholesterol. LDL receptor synthesis declines rapidly as does de novo synthesis of cholesterol.

However, even when contact-inhibited, ECs probably retain the ability to take up and degrade modified LDL. This has been demonstrated for sinusoidal capillary ECs but surprisingly aortic ECs did not appear to take up labelled acetylated and acetoacetylated LDL in rats, dogs or guinea pigs.[203] Possibly the sinusoidal cells are specialised for direct uptake of modified LDL from the plasma while large vessel endothelial cells are less efficient. Smooth muscle cells, fibroblasts and other non-endothelial cells (except parenchymal cells from sinusoidal organs) may not be exposed to such high concentrations of LDL as endothelial cells, which probably transport it freely into the intima. But here LDL appear to be prevented from penetrating the arterial wall in high concentration further than the internal elastic lamina, which is impermeable to LDL.[262] LDL may also partition into the basement-membrane matrix: the basis of its selective retention may be a specific interaction with proteoglycans.[19] Such a partitioning effect would go some way towards explaining the the finding of Smith and Staples[262] of intimal LDL at twice the plasma concentration in normal human artery. LDL within the intima might be modified by macrophages and ECs thus becoming degradable via scavenger receptor-mediated endocytosis.

Receptor Distribution on Active Cells

The LDL receptor distribution on normal human skin fibroblasts and on subconfluent ECs was mapped at the ultrastructural level with the receptor probes Au-LDL and C7 monoclonal antibodies . In both cell types, two distinct patterns of receptors were seen: one monodisperse and interspersed amongst the other clustered pattern. The clustered pattern represented receptors grouped in coated pits. As alluded to in Chapter 4 (pages 128 and 159-162), the present finding provides ultrastructural evidence that a significant proportion of LDL receptors are scattered randomly on the surface of fibroblasts. This possibility was discussed in a a review by Goldstein et al.[97] A new finding reported here is the existence of the same two patterns of LDL receptors on the surface of endothelial cells. The present study demonstrates these two populations by direct topographical visualisation of the cell surfaces in whole-mounted cells where previously the less convincing thin section approach had been used.[6]

The significance of the diffuse population of receptors can only be speculated upon at present. It may represent newly-recycled receptors which have yet to be clustered into coated pits. This interpretation contrasts with that of Robenek et al.[215] who found only the clustered pattern. It

might be argued that the random distribution reported here was artefactual, but the additional finding of only randomly distributed receptors on the mutant fibroblast GM 2408A The receptors of which are known from other studies[7] to lack the ability to cluster) suggests that the patterns reported here for normal cells, reflect the actual receptor distributions at the time of analysis.

Epilogue

Although this thesis is ostensibly concerned with the cell biological phenomenon of receptor-mediated endocytosis of LDL by endothelial cells, its ultimate concern is with atherogenesis. It is the consuming interest of thousands of medical researchers to contribute some new elements to the increasingly complex models for atherosclerosis. The aetiology of atherosclerosis has yet to be comprehensively defined owing to the large number of associated factors. The cause of a phenomenon has been defined £ as "the antecedents which it invariably and necessarily (or unconditionally) follows, provided there are no counteracting conditions among them." From this it emerges that causation rarely relies upon a single factor but rather an array of factors which act together. This is particularly likely in such complex systems as those which produce atherosclerosis. With the progress of research, factors which are invariably associated with a phenomenon become clear. They are elevated to causal status and it is from these that mechanisms are postulated and conceptual models built. Usually these models collapse to a greater or lesser extent under the intense verificatory processes which characterise science. New factors emerge from the use of better techniques and the cycle continues.

Two clusters of factors can be resolved when considering a mechanism for atherogenesis: the first is the response of vascular and peripheral cells to lipid overload (in particular cholesterol) while the second concerns factors which cause persistently high circulating levels of lipid. The insights from the study of lipid metabolism of mutant cells have highlighted the first while environmental factors focus on the second. The contribution of the endothelial cell is consistent with its boundary nature: it "sits on the fence" and controls access to the subendothelium, which with its extracellular matrix and internal elastic lamina is truly the "fence"! In response to lipid overload, the normal endothelium may modify and degrade LDL and, on the other hand if injured itself, it may lose its barrier function. Both responses may be atherogenic.

Like cancer, atherosclerosis is probably a general response to a number of possible insults and genetic defects. If this is true, then the study of atherogenesis will lead to the better understanding of a number of biological subsystems, e.g. the cell biology of the vascular cells. Conversely, independent understanding of the normal cell biology of vascular cells will shed light on the pathobiology of atherosclerosis. Only a multidisciplinary attack on the problem (which is very much underway) will speedily bring the understanding needed to alleviate or even prevent the disease in man and animals.

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